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Cyclosporine A Effects on Gingival Fibroblasts and Comparison of Some of Its Effects with Nifedipine and Phenytoin *in vitro*

BY

XUE FEN FAN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN
PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
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IN
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The undersigned certify that they have read, and recommend to the Faculty of Gradute Studies and Research for acceptance, a thesis entitled "Cyclosporine A Effects on Gingival Fibroblasts and Comparison of some of its Effects with Nifedipine and Phenytoin *in vitro*" submitted by Xue Fen Fan in partial fulfilment of the requirements for the degree of Master of Science.

Paul Hart

Supervisor

Date: January 16, 1990.

For Datong and my parents

ABSTRACT

The side-effects of cyclosporine A (CsA), nifedipine and phenytoin on gingiva are very similar. The pronounced feature of the drug-associated gingival hyperplasia is an increase in collagen content in the tissue. The aims of the studies were to investigate the cellular mechanism of drug-associated gingival hyperplasia by testing the effects of cyclosporine A on some of the biological functions of human gingival fibroblasts, and to compare some of the effects with those of nifedipine and phenytoin.

The studies were carried out by culturing human gingival fibroblasts explanted from non-CsA-treated normal gingival tissue and CsA-treated patients' tissue with or without hyperplasia (CsA-responder or CsA-non-responder cell strains). The results showed that cyclosporine A, nifedipine and phenytoin did not affect proliferation of fibroblasts. Cyclosporine A had no effect on $[Ca^{2+}]i$. Cyclosporine A slightly enhanced phagocytosis of latex beads. Cyclosporine A was rapidly taken up into the cells and highly concentrated in the cytosol. Ouabain did not affect this process. The high affinity cytosolic CsA-binding site has a K_d of 2.6×10^{-7} M. Although cyclosporine A did not inhibit collagenase activity per se, it reduced concanavalin A (ConA)- or human recombinant interleukin 1α (IL-1α)-, but not hrIL-1ß-, stimulated collagenase production by fibroblasts. Cyclosporine A inhibited the degradation of fibrillar collagen by fibroblasts in a dose-dependent manner in all the strains tested. Nifedipine or phenytoin were weaker than cyclosporine A in normal cell strains, while neither nifedipine nor phenytoin had this effect in the CsA-responder cell strains. However, nifedipine and phenytoin both inhibited ConA-induced collagenase production by both normal and CsA-responder cells. Unlike cyclosporine A, which inhibited all the cell strains tested, this effect of nifedipine or phenytoin was cell strain-dependent.

Our results suggest that cyclosporine A, nifedipine or phenytoin can directly act on the fibroblasts but the precise molecular mode of action of each of these drugs may be different. By being passively transported into the cells and concentrated in the cytosol, cyclosporine A most likely acts in the cytoplasm. We concluded that cyclosporine A, nifedipine or phenytoin can depress degradation of collagen by fibroblasts to a certain extent. This could partially explain the collagen accumulation in these drug-associated gingival hyperplasia tissue.

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LIST OF ABBREVIATION

A23187, an antibiotic and a calcium ionophore.

Ala, alanine.

AMP, adenosine monophosphate.

APC, antigen presenting cells.

ASC, acid soluble collagen.

ATPase, adenosine triphosphatase.

[Ca^{++}]i, intracellular free calcium ion concentrations.

CaM, calmodulin.

CsA (or CSA), cyclosporine A, or ciclosporin A.

[^{3}H]-CsA, tritiated CsA.

CsA-GH, CsA-induced gingival hyperplasia.

CsH, cyclosporine H.

ConA, concanavalin A.

DMEM, Dubecco's modified Eagle medium.

DMEM-0, serum free DMEM.

DMEM-10, DMEM + 10% fetal calf serum.

DMSO, dimethyl sulfoxyide.

dpm (or DPM), disintegrations per minute.

EGTA, ethylene glycol bis (ß-aminiethylether)-N,N,N',N'-tetraacetic acid.

FCS, fetal calf serum.

FITC, fluorescein isothiocyanate.

Fura 2, 1-[2-(5-carboxyoxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid.

GAG, glycosaminoglycans.

GH, gingival hyperplasia.

Gly, Glycine.

GMP, guanine monophosphate.

HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

hrIL-1α or ß, human recombinant interlukin-1 alpha or beta.

hrTNF-(α)/(ß), human recombinant tumor necrosis factor alpha or beta.

Ile, isoleucine.

IL-1, interleukin-1.

IL-2, interleukin-2.

K_d, dissociation constant.

LAF, lymphocyte activation factor.

Leu, leucine.

LPS, lipopolysaccharide.

LSC, liquid scintillation counter.

MMP(s), matrix metalloproteinase(s).

NF, nifedipine.

NF-GH, nifedipine-induced gingival hyperplasia.

NS, non-statistical difference.

PBS, phosphate-buffered saline.

PDC, potential-dependent channel.

PDE, phosphodiesterase.

PHA, phytohemagglutinin.

PHT, phenytoin.

PHT-GH, phenytoin-induced gingival hyperplasia.

PMA, phorbol-12-myristate-13-acetate.

PMN, polymorphonuclear (leukocytes).

PPI, peptidyl prolyl cis/trans isomerase.

PS, physiological saline.

Quin 2, 2-{[2-bis(carboxymethyl)-amino-5-methylphenoxy]-methoxy-8-bis-(carboxymethyl)-aminoquinoline.

ROC, receptor-operated channel.

R.T., room temperature.

S.A., specific activity.

S.D., standard deviation.

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

W7, [N-(6-aminohexyl)-5-chloronaphthalene-1-sulfonamide], a calmodulin inhibitor.

TIMP, tissue inhibitor of metalloproteinases.

TPA, 12-O-tetradecanoylphorbol-13-acetate.

Val, valine.

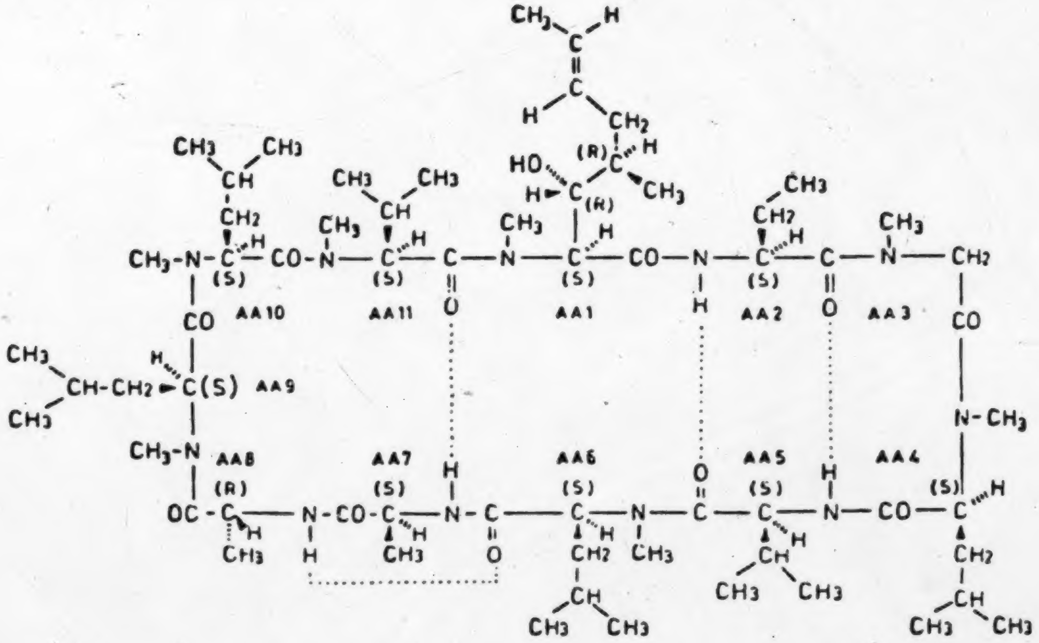
CHAPTER I

GENERAL INTRODUCTION

1.1 BACKGROUND

Cyclosporine A (CsA) is a metabolite of the fungus, *Tolypocladium inflatum Gams* (Borel, 1982). It is a cyclic undecapeptide (Figure 1.1) and seven of its amino acids are hydrophobic (Wenger, 1982). It is widely used in the clinic as an immunosuppressive drug, for the prevention of organ rejection (Calne et al., 1981) and treatment of some kinds of autoimmune disease (Feutren et al., 1986). The side-effects of CsA treatment at therapeutic concentrations include nephrotoxicity, hepatotoxicity, hirsutism, arteriolar and venous thrombosis, and gingival hyperplasia (Bennett and Norman, 1986). This CsA-induced gingival hyperplasia (CsA-GH) resembles the much longer studied phenytoin- (PHT) and recently found nifedipine-(NF) induced gingival hyperplasia (PHT-GH, NF-GH) (Butler et al., 1987).

CsA is biotransformed in liver. The metabolites are excreted mainly by biliary excretion and a small amount by urinary elimination (Wood et al., 1983). The primary metabolites found in human urine are metabolites 1, 17 and 21 (Figure 1.2) (Lemaire et al., 1986). The major metabolites in urine are the primary metabolite 17 and its secondary



Hydrogen bonds:

NH (α-aminobutyric acid) to C=O (Val-5);

NH (Val-5) to C=O (aminibutyric acid);

NH (Ala-7) to C=O (MethylVal-11);

NH (D-Ala-8) back to the C=O (MethylLeu-6).

Figure 1.1 Chemical structure of cyclosporine A ($C_{62}H_{111}O_{112}$; M.W. 1202 Dalton). Cyclosporine H, an immune inactive analog of cyclosporine A, has a L to D stereoisomerization of methylvaline at position 11 (Wenger, 1982; Wartburg and Traber, 1986).

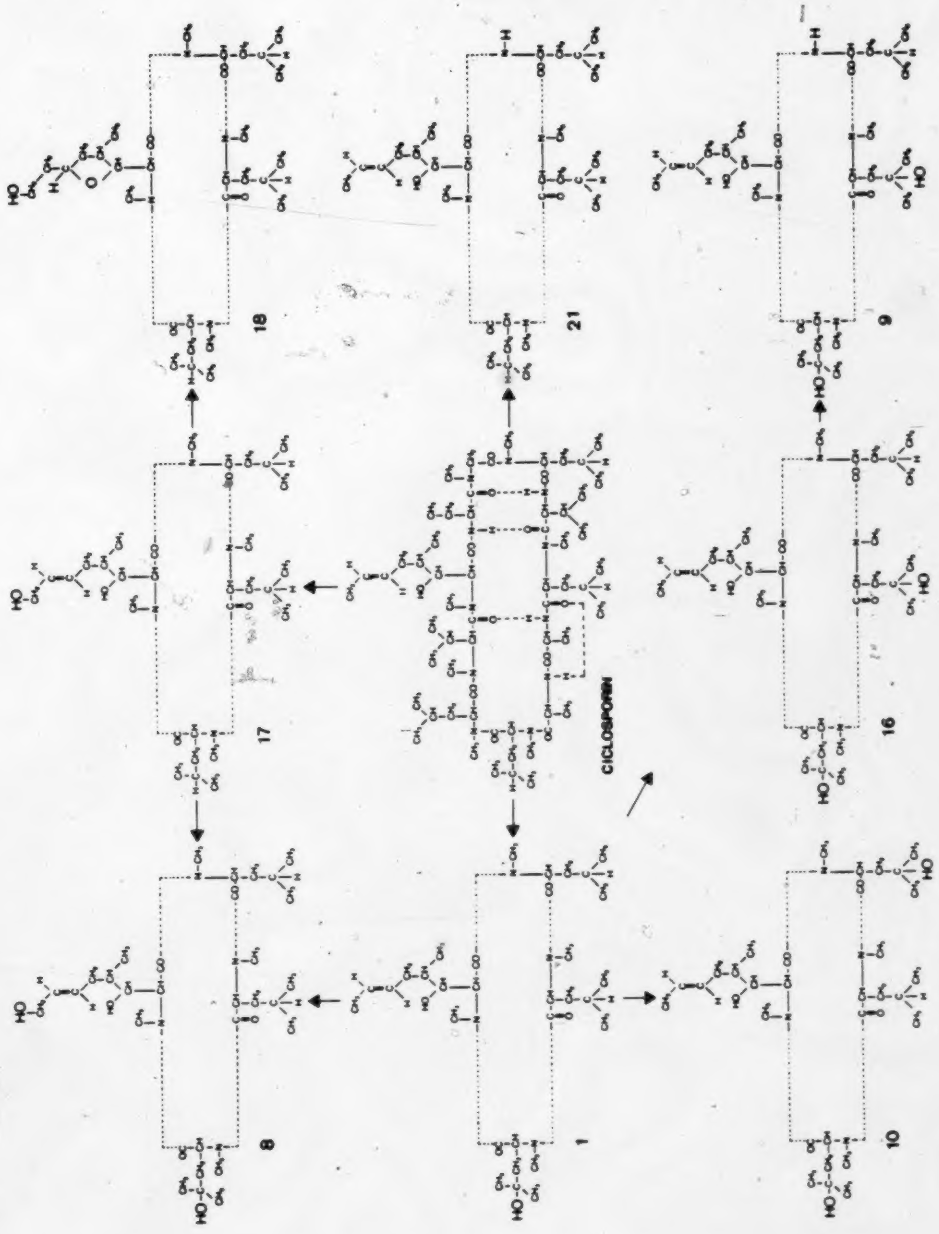


Figure 1.2 The biotransformation of cyclosporine A *in vivo*.

metabolite 8 (Lemaire et al., 1986). Transformation reactions of CsA are restricted to N-demethylation, hydroxylation and cyclization of 4 amino acids, namely 1, 4, 6 and 9 (Maurer et al., 1983) (Figure 1.2). All the metabolites preserve the cyclic oligopeptide structure of CsA. They are still hydrophobic but have sufficient water-solubility to be eliminated from bile or urine (Lemaire et al., 1986). The immunosuppressive activity of all of the metabolites is very low and should not have a significant effect on the immunosuppressive treatment in vivo (Lemaire et al., 1986). The therapeutic serum levels of CsA are recommended to be 100 to 300 ng/mL (Bowers and Canafax, 1984).

Phenytoin (5,5-diphenylhydantoin, or dilantin) is an anticonvulsant drug which is most often used in the control of epileptic seizures (Goodman and Gilman, 1975). Nifedipine is a dihydropyridine and is an increasingly popular medication applied in the treatment of vasospastic angina, chronic stable angina and ventricular arrhythmias (Karlsberg and Aronow, 1982). The chemical structures of NF and PHT are shown in Figures 1.3 and 1.4, respectively (Triggle, 1982; Goodman and Gilman, 1975).

The therapeutic serum levels of PHT and NF are 10 to 30 µg/mL and 0.025 to 0.1 µg/mL, respectively (Drug: Facts and Comparison, 1987).

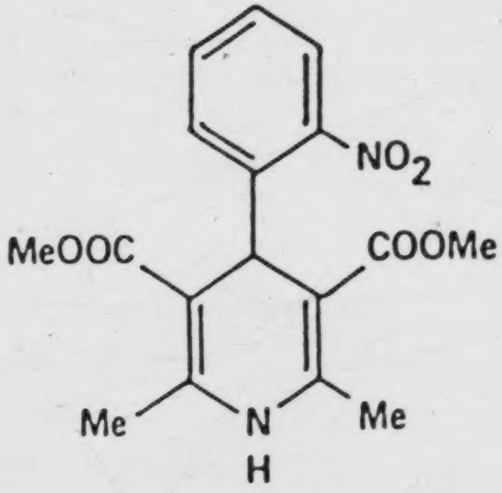


Figure 1.3 Chemical structure of nifedipine.

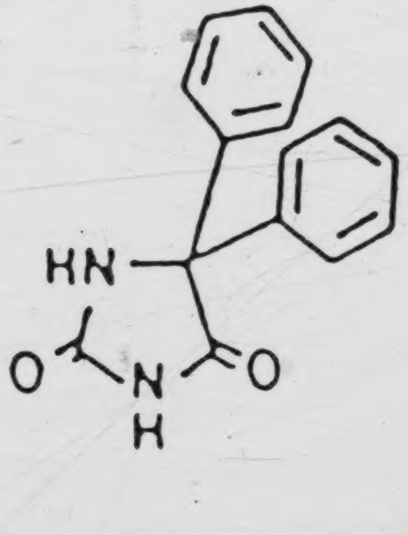


Figure 1.4 Chemical structure of phenytoin.

1.1.1 The problem: Cyclosporine A-, nifedipine- and phenytoin-induced gingival hyperplasias.

During CsA treatment, about 70% of the CsA recipients develop gingival hyperplasia (GH) (Daley et al., 1986). For PHT-GH, it was reported that the incidence was 0 to 84.5% (Angelopoulos and Goaz, 1972). This great variance is probably due to the different number of patients observed and non-standardized criteria for grading of the hyperplasia (Angelopoulos, 1975). Most authors estimate that the incidence of PHT-GH is about 40% to 50% (Angelopoulos, 1975; Hassell, 1981). There is no information in the literature on the incidence of NF-GH.

The mechanism of these drug-associated gingival hyperplasias (GH) is unknown. The clinical manifestations, histopathological characteristics and treatment of these three types of drug-associated GH are very similar (Livingston, 1970; Shafer et al., 1983; Wysocki et al., 1983; Rateitschak-Plüess et al., 1983; Lucas et al., 1985; Rostock et al., 1986; Delilers et al., 1986; Barak et al., 1987; Butler et al., 1987). It is a chronic process and usually occurs after one to several months' administration of the drugs. To the naked eye, it appears as nodular processes in the gingivae, usually starting on the labial surface of interproximal papillae, more commonly in association with the anterior teeth. The gingival tissues are dense, resilient and usually show no tendency to bleed. All three types of drug-associated GH usually exhibit

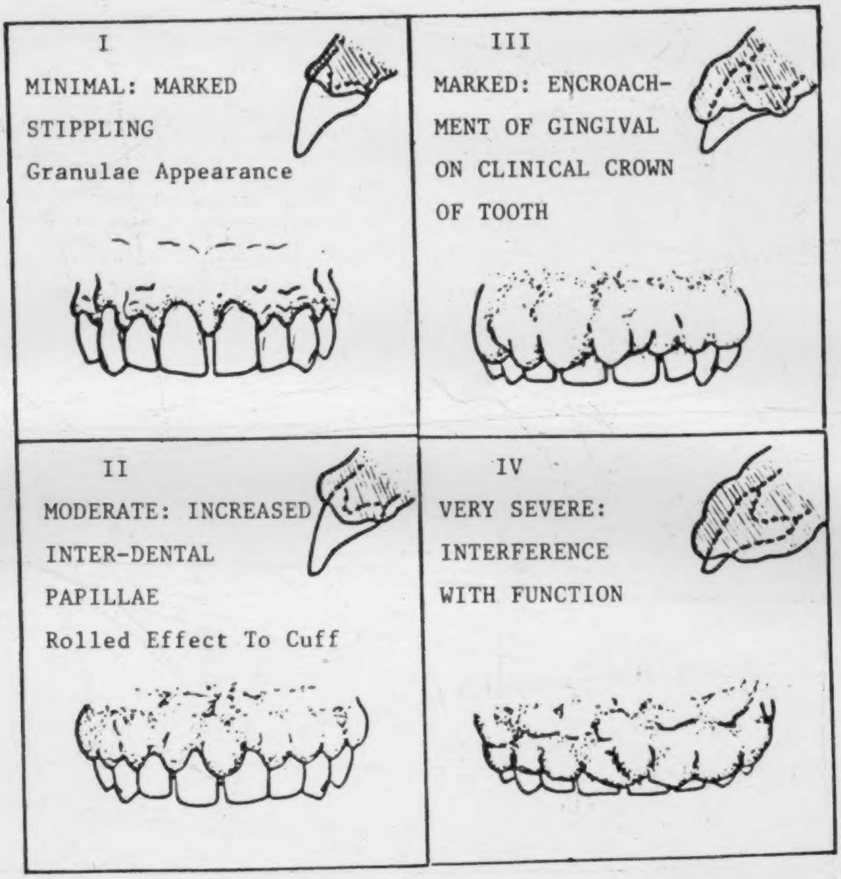


Figure 1.5 Diagrammatic illustration of the degrees of gingival hyperplasia.

a fibroepithelial hyperplasia. There is a thickening of stratified squamous epithelium, mainly in the spinous layer, with slight to moderate parakeratosis. Some areas of the epithelium show attenuated and elongated "rete peg" ridges, which penetrate the underlying lamina propria. In the subepithelial connective tissues, few or many fibroblasts have been reported in different case reports. Collagen content increase is one of the major features and it is sometimes in the form of irregularly arranged dense bundles. A slight to moderate infiltration or focal accumulation of inflammatory cells, consisting primarily of plasma cells is not unusual. The severity of the GH can be graded into 4 degrees (Figure 1.5) (Panuska et al., 1960). The treatment procedures for all these three types of drug-associated-GH include the maintenance of good oral hygiene; and, if it is severe, surgical excision of overgrown gingival tissue. Recurrence is common but natural recession occurs after terminating CsA, NF or PHT treatment.

1.1.2 Pharmacology of cyclosporine A, nifedipine and phenytoin.

It is useful to understand the pharmacology of CsA, NF or PHT before investigating their side-effects on gingiva. However, the immunology of lymphocyte activation, transplantation rejection and autoimmune disease are very complicated issues and not well understood yet, neither are the pharmacological effects of CsA on these systems.

The effect of CsA in the immune system mainly involves the inhibition of the early events of lymphocyte activation (Klaus and Chisholm, 1986). Effects of CsA on T lymphocytes include blocking interleukin-2 (IL-2) and gamma-interferon production at the mRNA level in helper T lymphocytes and IL-2 receptor formation in cytotoxic T lymphocyte membranes. However, CsA does not affect the functionally active macrophages and cytotoxic T cells. It is still controversial whether CsA can interfere with the function of antigen presenting cells (APC) (Borel, 1988). Recently, Yoshimura et al. (1988) reported that human peripheral blood lymphocytes from patients treated with CsA (100 to 200 ng/mL in serum level) showed significant reduction of gamma-interferon, IL-1 and IL-2 production. It is now established that CsA is not only a T lymphocyte selective agent, it can affect B cells, fibroblasts and epithelial cells (Aszalos, 1988).

Its precise mode of action at subcellular and molecular levels remains unclear. There are no specific receptors for CsA in cell membranes (LeGrue et al., 1983). However, it can slightly decrease the fluidity of the cell membrane (Matyus et al., 1986). To locate the possible site of CsA action, its effects on some processes which are involved in the normal lymphocyte activation have been studied. CsA does not affect inositol phosphate breakdown, cyclic nucleotide regulation and phosphokinase C activity (Aszalos, 1988). Although CsA binds to cyclophilin with high affinity (Handschumacher et al., 1984), this protein was recently characterized as a non-specific enzyme: peptidyl prolyl cis/trans isomerase (PPI) (Schmid et at., 1989). Thus, the pharmacological significance of CsA-cyclophilin binding is unknown. Colombani et al. (1985) reported that CsA binds to calmodulin (CaM), an intracellular Ca^{2+} regulatory protein, and inhibits calmodulin-activated phosphodiesterase (PDE) activity in vitro. The argument against CsA-CaM binding being relevant to its action is that the immunologically inactive CsA analog cyclosporine H (CsH) also binds to CaM with a similar affinity to that of CsA (LeGrue et al., 1986). The binding of CsA to CaM does not inhibit the activity of PDE, nor affect smooth muscle contraction which is related to the Ca^{2+}-CaM-dependent myosin light chain kinase (LeGrue et al., 1986). It is postulated that CsA probably binds to Ca^{2+}-calmodulin complex via a hydrophobic interaction and stabilizes it. This CsA-Ca^{2+}-calmodulin complex may be functionally inactive in some processes (P.G. Scott, personal communication). The responsiveness of events in lymphocyte activation to CsA is stimulating-agent dependent. The present results from the literature suggest that if the stimulating path of lymphocyte activation is Ca^{2+} influx-dependent, such as the proliferation of T cells in response to the stimulation by ConA (concanavalin A), PHA (phytohemoagglutinin) and calcium ionophore A23187, it is CsA-sensitive; whereas, if it is through the protein kinase C pathway, for example, LPS-(lipopolysaccharide) or phorbol ester-stimulated T cell activation, it is relatively CsA-resistant (Aszalos, 1988). However, there is evidence that CsA does not directly affect intracellular free calcium ion concentrations ([Ca^{2+}]i) in primary cells nor the A23187-induced Ca^{2+} influx (Aszalos et al., 1987). Only under certain circumstance, such as pretreatment of lymphocytes with CsA (1 μg/mL) for 30 min, does it inhibit [Ca^{2+}]i changes during PHT activation (Gelfand et al., 1987). Biologically inactive CsH does not interfere with this effect (Aszalos, 1988). There is thus considerable disagreement on this issue. It could be clarified by re-examining the [Ca^{2+}]i in different cell activation modes. It is not clear whether the effects of CsA at all of the above cellular and molecular action points are related to its pharmacological action.

NF is a calcium channel blocker and its effect on myocardial cells and vascular smooth muscle cells is to cause relaxation due to electromechanical uncoupling (Triggle, 1982). The process of calcium mobilization in a cell usually involves the activation of two discrete calcium channels (Bolton, 1979; Meisheri et al., 1981). One is the potential-dependent channel (PDC), which is activated by membrane depolarization and initiates a process of electromechanical coupling. The other is a receptor-operated channel (ROC) activated by membrane receptors that are independent of membrane potential. The latter may be related to the pharmaco-mechanical coupling process. Unlike some divalent or trivalent cations (Mn^{2+}, Co^{2+}, La^{3+}) which can bind to the calcium channel and thus block it, NF is a neutral molecule that is unlikely to act via binding to the channels (Rosenberger et al., 1979). NF is believed to act on the PDC (Triggle, 1982). More precisely, NF blocks the "slow inward current" of a cardiac cell, i.e., the current when the cell is depolarized from -20 to 0 mV during the action potential (Antman et al., 1980). The cellular membrane channels carrying this slow inward current are also termed "calcium channel" since the major ionic species crossing the cell membrane during this period is Ca^{2+} (Reuter, 1979; Beeler and Reuter, 1977).

It is well established that PHT blocks sodium channels in a manner similar to that of tetrodotoxin and thus shows a marked reduction of sodium influx in the nerve membrane (Woodbury, 1982a). This effect may be potential-dependant (Schwartz and Vogel, 1977). PHT also has a marked inhibitory effect on calcium influx in synaptosomes, either directly acting on calcium transport or acting secondarily to the decrease in passive sodium influx (Baker and Blaustein, 1968; Blaustein and Weismann, 1970; Lowe et al., 1976). PHT can increase Na^+-K^+ transport in epithelial cells, probably due to activation of the Na^+-K^+-ATPase or increase in its synthesis (Woodbury, 1982a). Several neurotransmitters are affected by PHT (Woodbury, 1982a). For example, PHT deceases the uptake of norepinephrine as well as inhibiting its secretion, a process which is Ca^{2+}-dependent. It may also suppress the degradation of norepinephrine by monoamine oxidase. The uptake of amino acid by the synapse is increased, a process subsequent to the sodium gradient increase across the cell membrane. The effect of acetylcholine on the increase of sodium permeability of postsynaptic membrane is also inhibited by PHT. The marked membrane-stabilizing effect of PHT may be responsible for its anticonvulsant action (Woodbury, 1982a).

After absorption into the circulatory system and entrance into tissue, PHT is rapidly and reversibly bound to proteins. About 90% of PHT in central nerve system binds to brain tissue (Woodbury, 1982b). The binding of PHT to protein is relatively non-specific (Goldberg, 1980). At the subcellular level, PHT can bind to the microsomal fraction of brain homogenates (Woodbury, 1980). It also shows an inhibition of protein synthesis in brain tissue in in vivo studies (Jones and Woodbury, 1976). The polymerization of microtubules is also inhibited by the drug (MacKinney et al., 1975). Another important intracellular effect of PHT is that it selectively inhibits cyclic AMP and cyclic GMP elevation caused by some agents that induce cellular depolarization, such as ouabain, veratridine, glutamate and high concentrations of K^+ (Ferrendelli, 1980). Whether all the above effects of PHT on brain tissues are related to its anticonvulsant activity is not clear.

1.1.3 Review of research on drug-associated gingival hyperplasia: The direct effects of cyclosporine A, nifedipine and phenytoin on gingival fibroblasts.

The most often used model adopted for the studies of these drug-associated gingival hyperplasias in vitro is to culture gingival fibroblasts explanted from normal human individuals or CSA-, NF- or PHT-treated patients or animals under defined conditions. Some biological and biochemical parameters which are directly or indirectly relevant to collagen metabolism can be obtained.

There are reports that PHT has no effect (Keith et al., 1977), decreases (Noess, 1969) or increases (Rose and Robertson, 1977) the proliferation of fibroblasts. Fibroblasts from PHT-GH responder cells strains showed elevated levels of total protein synthesis in culture in the absence of PHT (Hassell et al., 1976; Benveniste and Bitar, 1980). About 20% of the total newly synthesized protein is collagen (Hassell et al., 1976) and this is due to an increased steady state level of collagen mRNA (Narayanan et al., 1988). The synthesis of GAG (glycosaminoglycans) by the PHT-GH responder cells was also increased in the absence of PHT, as measured by $^{35}SO_4^{2-}$-incorporation into the cultured fibroblasts (Kantor and Hassell, 1983). There were reports showing that both the degradation of collagen by gingival tissue and the production of collagenase by the PHT-GH responder cells or PHT-treated normal fibroblasts were reduced (Goultschin and Shoshan, 1980; Hurum et al., 1982; Zebrowski et al., 1988). Hassell (1982) also reported that collagenase secreted by the fibroblasts from PHT-GH responder cell strains was mainly inactive. The above results on collagen catabolism are also supported by electron microscopy studies which showed that the numbers of phagolysosomes were decreased (Hall and Squier, 1982).

So far in the literature, experimental pathology studies of CsA-GH or NF-GH are fewer than those of PHT-GH, but some similar results have been seen. Both increases and decreases in proliferation of fibroblasts were reported (Zebrowski et al., 1988; Zebrowski and Singer, 1989). For effect of CsA on collagen synthesis, both increases and decreases have been seen (Coley et al., 1986; Tipton and Dabbous, 1986). The synthesis of the constituents of extracellular matrix in CsA-treated fibroblasts is also increased (Zebrowski and Singer, 1989). The phagocytosis of collagen fragments by fibroblasts in CsA-GH tissue was decreased (McGaw and Porter, 1988). Zebrowski et al. (1988) showed that CsA (250 ng/mL and 500 ng/mL) had no effect on collagenase activity, while NF (100 and 200 ng/mL) increased its activity. Tipton et al. (1989) showed that the CsA decreased collagenase activity in the medium from some fibroblasts cultures in a dose-dependent manner. In a recent report, CsA (100 to 200 ng/mL) was not found to affect the cellular cyclic AMP level in human gingival fibroblasts (Metzler et al., 1989). CsA did not directly interfere with the production of prostaglandin E by human gingival fibroblasts in vitro (Roberts et al., 1989). Although more and more information on drug-associated GH has been accumulated, little is known about the precise mechanism of CsA-GH, NF-GH or PHT-GH.

1.2 HYPOTHESIS

The work discussed above suggests that CsA, NF or PHT directly interfere with gingival fibroblast metabolism. In addition to its effect on collagen synthesis (see above), CsA may also inhibit collagenase production under certain circumstances, thus contributing in more than one way to collagen accumulation in gingiva. Furthermore, for regulation of collagenase production, we propose that calcium influx may serve as a signal in the fibroblasts. CsA may interfere with this process. NF or PHT may affect collagen metabolism in a similar manner. At least, they are similar in outcome even if their intracellular mechanisms differ.

1.3 PURPOSE AND STRATEGY OF THE STUDY

This study was designed to test for certain direct effects of CsA, and to a limited extent NF and PHT, on the functional properties of gingival fibroblasts. The following experiments were carried out: (i) the effect of CsA, NF or PHT on the proliferation of gingival fibroblasts; (ii) the effect of CsA on the phagocytosis of latex beads by the cells; (iii) the effect of CsA on intracellular calcium concentrations ($[Ca^{2+}]i$) and on calcium ionophore-induced $[Ca^{2+}]i$ changes; (iv) the kinetics of CsA uptake, distribution of CsA and the CsA-binding molecule(s) in the fibroblasts; (v) comparison of effects of NF or PHT with CsA on the degradation of fibrillar collagen; and (vi) the effects of CsA, NF or PHT on stimulation of collagenase production.

The first two steps in the studies would help us to understand whether CsA can simply interfere with obvious biological functions of fibroblasts, such as cell proliferation and phagocytosis. Since NF and PHT are believed to affect calcium channels in myocardial cells and nerve cells respectively (see above), it would be interesting to know whether CsA can interfere with $[Ca^{2+}]i$ in fibroblasts. If it does, it would suggest that these drugs have a common mode of action in drug-associated GH. Then we moved on to study the kinetics of uptake of CsA and to localize the possible CsA-binding molecule(s) in the cell. This would give us an idea whether CsA can directly affect the fibroblasts. Finally, we focus on the studies of the effect of CsA, NF or PHT on the degradation of collagen fibrils and collagenase activities in the medium.

1.4 CONTRIBUTION AND SCOPE

Through this project, we hope to contribute to a better understanding of the effects of CsA, NF and PHT on gingival fibroblasts, especially on the degradation of collagen. We also expect to learn of the similarities and differences in the effect of CsA, NF and PHT on the catabolism of collagen by the fibroblasts. Thus it would help us to explain the mechanism of these drug-associated GH at a cellular physiology level, but not at the precise molecular biology level.

One of the very important things that is still not understood is the precise molecular mechanism of regulation of collagenase production in vitro or in vivo. Although it is a critical point in studies of the mechanism of these drug associated-GH, the problem is too complicated to be solved systematically in a short period of time. In the following paper (Chapter II), we study only one of these possible action modes potentially related to the regulation of collagenase production, i.e. the relationship of $[Ca^{2+}]i$ to CsA action, which is possibly an action mechanism, if they are the same, common to all three drugs (see above). If we cannot prove this point, we do not intend to go on any further in this direction.

Collagen metabolism involves the balance of collagen synthesis and catabolism in vivo. Although fibroblasts are the major source of collagenase which is most important to the collagen metabolism in soft connective tissue, other types of cell such as epithelial cells, polymorphonuclear (PMN) leukocytes and macrophages or monocytes also contribute to collagenase production under certain circumstance (for a review, see Woolley, 1984). Other indirect factors, such as the inhibition of IL-1 production in monocytes by CsA at low concentration (50 ng/mL) (Tipton et al., 1989) may also play a significant role in the drug-associated GH.

We do not intend to cover every aspect of all the possible action points of CsA. We would not compare every effect of CsA on fibroblasts tested with NF and PHT either. However, by combining with other published results, we believe it would give us a more comprehensive picture of the CsA-GH story and some more information on NF-GH and PHT-GH.

1.5 BIBLIOGRAPHY

ANGELOPOULOS, A.P., GOAZ, P.W. Incidence of diphenylhydantoin gingival hyperplasia. Oral Surg. 34:898-906, 1972.

ANGELOPOULOS, A.P. Diphenylhydantoin gingival hyperplasia, A clinicopathological review. 1. Incidence, clinical features and histopathology. J. Can. Dent. Assn. 41: 103-106, 1975.

ANTMAN, E.M., STONE, P.H., MULLER, J.E. BRAUNWALD, E. Calcium channel blocking agents in the treatment of cardiovascular disorders. Part I: Basic and clinical electrophysiologic effects. Ann. Internal Med. 93:875-885, 1980.

ASZALOUS, A., DAMJANOVICH, S., TRON, L. Effect of cyclosporine A and ionophores on the intracellular pH of lymphocytes as measured by flow cytometry. Biophys. J. 51:524a, 1987.

ASZALOS, A. Cyclosporine: Some aspects of its mode of action. A review. J. Med. 19:297-316, 1988.

BAKER, P.F., BLAUSTEIN, M.P. Sodium-dependent uptake of calcium by crab nerve. Biochim. Biophys. Acta.

150:167-170, 1968.

BARAK, S., ENGELBERG, I.S., HISS, J. Gingival hyperplasia caused by nifedipine. Histopathologic findings. J. Periodontol. 58:639-642, 1987.

BEELER, G.W., REUTER, H. Reconstruction of the action potential of ventricular myocardial fibres. J. Physiol. (Lond). 268:177-210, 1977.

BENNETT, W.M., NORMAN, D.J. Action and toxicity of cyclosporine. Ann. Rev. Med. 37:215-224, 1986.

BENVENISTE, K., BITAR, M. Effects of phenytoin on cultured human gingival fibroblasts. In: Phenytoin-induced Teratology and Gingival Pathology. T.M. Hassell, M. Johnston, K. Dudley, editors. Raven Press, N.Y., 1980. pp. 199-214.

BLAUSTEIN, M.P., WIESMANN, W.P. The effect of sodium ions on calcium movements in isolated synaptic terminals. Proc. Natl. Acad. Sci. U.S.A. 66:664-671.

BOLTON, T.B. Mechanisms of action of transmitters and other substances on smooth muscle. Physiol. Revs. 59:606-718, 1979

BOREL, J.F. The history of cyclosporin A and its significance. In: Cyclosporin A, proceedings of an international conference. D.J.G. White, editor. Elsevier Biomedical Press, N.Y., 1982. pp. 5-15.

BOREL, J.F. Basic science summary. Transplantation Proceedings. 20 Suppl.:722-730, 1988.

BOWERS, L., CANAFAX, D.M. Cyclosporine: Experience with therapeutic monitoring (review). Therapeutic Drug Monitoring. 6:142-147, 1984.

BUCY, R.P. Alloantigen-specific suppressor T cells are not inhibited by cyclosporin A, but do require IL-2 for activation. J. Immunol. 137:809-813, 1986.

BUTLER, R.T., KALKWARKF, K.L., KALDAHL, W.B. Drug-induced gingival hyperplasia: phenytoin, cyclosporine and nifedipine. J. Am. Dent. Assoc. 114:56-60, 1987.

CALNE, R.Y., ROLLES, K., WHITE, D.J.G. THIRU, S., EVANS, D.B., HENDERSON, R., HAMILTON, D.L. BOONE, N., MCMASTER, P., GIBBY, O., WILLIAMS, R. Cyclosporin A in clinical organ grafting. Transplantation. Proc. 13:349-358, 1981.

COLEY, C., JARVIS, K., HASSELL, T.M. Effect of cyclosporine-A on human gingival fibroblasts in vitro. J. Dent. Res. 65 (Special issue):353, 1986.

COLOMBANI, P.M., ROBB, A., HESS, A.D. Cyclosporin A binding to calmodulin: a possible site of action on T-lymphocytes. Science. 228:337-339, 1985.

DALEY, T.D., WYSOCKI, G.P., DAY, C. Clinical and pharmacologic correlations in cyclosporine-induced gingival hyperplasia. Oral Surg. Oral Med. Oral Path. 62:417-421, 1986.

DAMJANOVICH, S., ASZALOS, A., MULHERM, S.A., SZOLLOSI, M., BALAZS, M., TRON, L.,,FULWYLER, N.J. Cyclosporin depolarizes human lymphocytes: Earliest observed effect on cell metabolism. Eur. J. Immunol. 17:763-768, 1987.

DELILERS, G.L., SANTORO, F., POLLI, N., BRUNO, E., FUMAGALLI, L. RISCIOTTI, E. Light and electron microscopic study of cyclosporin A-induced gingival hyperplasia. J. Periodontol. 57:771-775, 1986.

Drug: Facts and Comparison. E.K. Kastrup (founding editor). J.B. Lippincott Company, St Louis, Missouri, 1987.

pp. 520 and 1121.

FERRENDELLI, J.A. Phenytoin: Cyclic nucleotide regulation in the brain. Adv. Neurol. 27:429-433, 1980.

FEUTREN, G., ASSAN, R., KARSENTY, G., DU ROSTU, H., SIRMAI, J., PAPOZ, L., VIALETTES, B., VEXIAU, P., RODIER, M., LALLEMAND, A. Cyclosporin increases the rate and length of remission in insulin-dependent diabetes of recent onset. Result of a multicentre double-blind trial. Lancet. 2:119-123, 1986.

GELFAND, E.W., CHEUNG, R.K., MILLS, G.B. Does cyclosporin inhibit lymphocyte activation at more than one site? J. Immunol. 138:1115-1120, 1987.

GOLDBERG, M.A. Phenytoin: Binding. Adv. Neurol. 27:323-337, 1980.

GOODMAN, L.S., GILMAN, A. The Pharmacological Basis of Therapeutics. MacMillan Publishing Co., Inc., N.Y., 1975. pp. 204-209.

GOULTSCHIN, J., SHOSHAN, S. Inhibition of collagen breakdown by diphenylhydantoin. Biochim. Biophys. Acta. 631: 188-191, 1980.

HALL, B.K., SQUIER, C.A. Ultrastructural quantitation of connective tissue changes in phenytoin-induced gingival overgrowth in ferret. J. Dent. Res. 61(7):942-952, 1982.

HANDSCHUMACHER, R.E., HARDING, N.W., RICE, J. DRUGGE, R.J. SPEICHER, D.W. Cyclophilin: A specific cytosolic binding protein for cyclosporin A. Science. 226:544-547, 1984.

HASSELL, T.M., PAGE, R.C., NARAYANAN, A.S. COOPER, C.G. Diphenylhydantoin (Dilantin) gingival hyperplasia: drug-induced abnormality of connective tissue. Proc. Natl. Acad. Sci., USA. 73:2902-2912, 1976.

HASSELL, T.M. Epilepsy and the oral manifestations of phenytoin therapy. Basel, Switzerland, Kruger Publishers. 1981, pp. 122-123.

HASSELL, T.M. Evidence for production of an inactive collagenase by fibroblasts from phenyltoin-enlarged human gingiva. J. Oral Pathol. 11:310-317, 1982.

HURUM, S., SODEK, J., AUBIN, J.E. Synthesis of collagen, collagenase and collagenase inhibitors by cloned human gingival fibroblasts and the effect of concanavalin A. Biochim. Biophy. Res. Commun. 107:357-366, 1982.

JONES, G.L., WOODBURY, D.M. Effects of diphenylhydantoin and phenobarbital on protein metabolism in the rat cerebral cortex. Biochem. Pharmacol. 25:53-61, 1976.

KANTOR, M.L., HASSELL, T.M. Increased accumulation of sulfated glycosaminoglycans in cultures of human fibroblasts from phenytoin-induced gingival hyperplasia. J. Dent. Res. 62:383-387, 1983.

KARLSBERG, P.R., ARONOW, W.S. Calcium channel blockers: indications and limitations. 1. Clinical pharmocology and use as antiarrhymic agents. Postgrad. Med. 72:97-110, 1982.

KEITH, D.A., PAZ, M.A., GALLOP, P.M. The effect of diphenylhydantoin on fibroblasts in vitro. J. Dent. Res. 56:1279-1283, 1977.

KLAUS, G.B., CHISHOLM, P.M. Does cyclosporine act in vivo as it does in vitro? Immunol. Today. 7:101-103, 1986.

LEGRUE, S.J., FRIEDMAN, A.W., KAHAN, B.D. Binding of cyclosporin by human lymphocytes and phospholipid vesicles. J. Immunol. 131:712-718, 1983.

LEGRUE, S.J., TURNER, R., WEISBRODT, N., DEDMAN, J.R. Does the binding of cyclosporin to calmodulin result in immunosuppression? Science. 234:68-71, 1986.

LEMAIRE, M., MAURER, G., WOOD, A.J. Pharmacokinetics and metabolism. In: Ciclosporin. J.B. Borel editor. Karger (Publisher), Basel. 1986. pp. 93-107.

LIVINGSTON, S. The medical treatment of epilepsy: Managing side-effects of anti-epileptic drugs. Pediatr. Ann. 8:261-266, 1970.

LOWE, D.A., RICHARDSON, B.P., TAYLOR, P., DONATSCH, P. Increasing intracellular sodium triggers calcium release from bound pools. Nature. 260:337-338, 1976.

LUCAS, R.M., HOWELL, L.P., WALL, B.A. Nifedipine-induced gingival hyperplasia. A histochemical and ultrastructral study. J. Periodontol. 56:211-215, 1985.

MCGAW, W.T., PORTER, H.B. Cyclosporine-induced gingival overgrowth: An ultrastructural stereological study. Oral Surg. Oral Med. Oral Pathol. 65:186-190, 1988.

MACKINNEY, A.A., VYAS, R. LEE, S.S. The effect of parahydroxylation of diphenylhydantoin on metaphase accumulation. Proc. Soc. Exp. Biol. Med. 149:371-375, 1975.

MATYUS, L., BALAZS, M., ASZALOS, A., MULHERM, S., DAMJANOVICH, S. Cyclosporin A depolarizes cytoplasmic membrane potential and interacts with Ca^{2+} ionophores. Biochim. Biophys. Acta. 886:353-360, 1986.

MAURER, G., LOOSLI, H.R., SCHREIER, E., KELLER, B. Disposition of cyclosporine in several animal species and man. I. Structure elucidation of its metabolites. Drug Metab. Dispos. 12:120-126, 1983.

MEISHERI, K.D., HUANG, O., VAN BREEMEN, C. Evidence of two separate Ca^{2+} pathways in smooth muscle plasmalemma. J. Mem. Biol 59:19-25, 1981.

METZLER, K., BEIRAGHI, S., SHANFELD, J. Effect of cyclosporin on cAMP level in cultured gingival fibroblasts. J. Dent. Res. 68 (Special issue):361, 1989.

NARAYANAN, A.S., MEYERS, A.S., PAGE, R.C. Regulation of collagen production in fibroblasts culture from normal and phenytoin-induced hyerplastic human gingiva. J. Periodont. Res. 23:118-121, 1988.

NOESS, T. The effect of 5,5-diphenylhydantoin (Dilantin) on fibroblast-like cells culture. J. Periodont. res. 4:163-164, 1969.

PANUSKA, H.J., GORLIN, R.J., BEARMAN, J.E., MITCHELL, D.F. The effect of anticonvulsant drug upon gingiva— A series of analyses of 1048 patients (I). J. Periodontol. 31:336-344, 1960.

RATEITSCHAK-PLÜESS, E.M., HEFTT, A., LÖRTSCHER, R., THIEL, G. Initial observation that cyclosporin-A induces gingival enlargement in man. J. Clinical Periodont. 10:237-246, 1983.

REUTER, H. Properties of two inward membrane currents in the heart. Annu. Rev. Physiol. 41:413-424, 1979.

ROBERTS, G., BEIRAGHI, S., SHANFELD, J., WILSON, S., CASAMASSIMO, P. Effect of cyclosporine A on prostaglandin E in human gingival fibroblasts. J. Dent. Res. 68 (Special issue):361, Abstract No. 1435, 1989.

ROSENBERGER, L.B., TICKU, M.K., TRIGGLE, D.J. The effects of

Ca^{2+} antagonists on mechanical responses and Ca^{2+} movements in guinea pig ileal longitudinal smooth muscle. Can. J. Physiol. Pharmacol. 57:333-347, 1979.

ROSE, G.G., ROBERTSON, P.B. Collagenolysis by human gingival fibroblasts cell lines. J. Dent. Res. 56:416-424, 1977.

ROSTICK, M.H., FRY, H.R., TURNER, J.E. Severe gingival overgrowth associated with cyclosporine therapy. J. Periodontol. 57:294-299, 1986.

SCHMID, F.X., KIEFHABER, T., SCHÖNBRUNNER, R. The mechanism of slow steps in protein folding. In: The 19th Federal of European Biochemical Societies Meeting. Rome, July 2-9, 1989. Abstract TU66L.

SCHWARTZ, J.R., VOGEL, W. Diphenylhydantoin: Excitability reducing action in single myelinated nerve fibers. Eur. J. Pharmacol. 44:241-249, 1977.

SHAFER, W.G., HINE, M.K., LEVY, B.M., TOMICH, C.E. Editors. A Textbook of Oral Patholgy. W.B. Saunders Company, Philadelphia, 1983. pp. 786-787.

TIPTON, D.A., DABBOUS, M.K. Heterogeneity of gingival fibroblast response to cyclosporine. J. Dent. Res. 65 (Special issue):331, 1986.

TIPTON, D., PABST, M., DABBOUS, M. Effect of immune response to Cs on fibroblast collagenase activity. J. Dent. Res. 68 (Special issue):361, 1989.

TIPTON, D.A., DABBOUS, M.K. Dose-dependent collagenolytic response profiles of gingival fibroblasts to cyclosporine. J. Dent. Res. 68 (Special issue):316, 1989.

TRIGGLE, D.J. Biochemical pharmacology of calcium blocker. In: Calcium Blockers: Mechanism of Action and Clinical Applications. S.F. Flain and R. Zelis, editors. Urban and Schwarzenberg, 1982. pp. 121-134.

WARTBURG, A.V., TRABER, R. Chemistry of the natural cyclosporin metabolites. In: Ciclosporin. J.F. Borel, editor. Karger (Publisher), Basel, 1986. pp. 28-45.

WENGER, R. Chemistry of cyclosporin. In: Cyclosporin A, proceedings of an international conference. D.J.G. White, editor. Elsevier Biomedical Press, N.Y., 1982. pp. 19-33.

WOOD, A.J., MAURER, G., NIEDERBERGER, W., BEVERIDGE, T. Cyclosporine: pharmacokinetics, metabolism and drug interactions. Transplant. Proc. 15 Suppl. 1:2409-2412, 1983.

WOODBURY, D.M. (1982a). Phenytoin: Mechanism of Action. In: Antiepileptic Drugs. D.M. Woodbury, J.K. Penry and C.E. Pippenger, editors. Raven Press, N.Y., 1982. pp. 269-281.

WOODBURY, D.M. (1982b). Phenytoin: Absorption, Distribution, Excretion. In: Antiepileptic Drugs. D.M. Woodbury, J.K. Penry and C.E. Pippemger, editors. Raven Press, N.Y., 1982. pp. 191-207.

WOODBURY, D.M. Phenytoin: Proposed mechanism of anticonvulsant action. Adv. Neurol. 27:447-471, 1980.

WOOLLEY, D.E. Mammalian collagenase. In: Extracellular Matrix Biochemistry. K.A. Piez and A.H. Reddi, editors. Elsevier Press, NY., Amsterdam, 1984. pp.119-157.

WYSOCKI, G.P., GRETZINGER, H.A., LAUPACIS, A., UAN, R.A., STILLER, C.R. Fibrous hyperplasia of gingiva: A side effect of cyclosporin A therapy. Oral Surg. Oral Med. Oral Path. 55:274-278, 1983.

YOSHIMURA, N., KAHAN, B.D., MATSUI, S., KITA, M., HAMASHIMA, T., OKA, T. Cyclosporine effect on immunoregulatory cells in kidney transplant recipients: Suppression of gamma-interferon and interleukin production. Transplantation Proceedings. 20 Suppl.:69-74, 1988.

ZEBROWSKI, E.J., RAMAMURTHY, N.S., SINGER, D.L., BRUNKA, J.R. Collagenase activity of cyclosporin, nifedipin and phenytoin treated fibroblasts. J. Dent. Res. 67 (Special issue): 331, 1988.

ZEBROWSKI, E.J., SINGER, D. Fibroblast function: Regulation by cyclosporin-induced hyperplastic gingiva conditioned growth medium. J. Dent. Res. 68 (Special issue):361, 1989.

CHAPTER II

EFFECTS OF CYCLOSPORINE A ON SOME OF THE BIOLOGICAL FUNCTIONS OF GINGIVAL FIBROBLASTS *IN VITRO*

2.1. INTRODUCTION

Cyclosporine A (CsA) is widely used as an immunosuppressive drug for the prevention of graft rejection. The side effects of CsA therapy include gingival hyperplasia, nephrotoxicity, hepatotoxicity, hirsutism, arteriolar and venous thrombosis, neurotoxicity and hypertension (Bennett and Norman, 1986). Gingival hyperplasia seems to occur to some degree in up to 70% of patients receiving CsA (Daley et al., 1986). Clinically and histopathologically, this condition resembles the much longer-studied phenytoin-associated (Wysocki et al., 1983) and the recently found nifedipine-associated, hyperplasia (Lucas et al., 1985). The cellular mechanism of the effect is unknown. Fibroblasts are believed to play an important role in CsA-associated gingival hyperplasia. Therefore, one approach adopted in a number of

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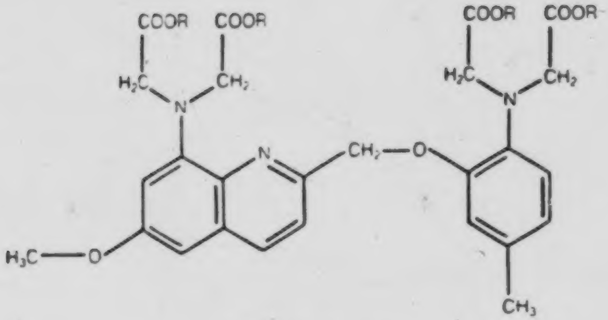
Abbreviation: EGTA, ethylene glycol bis (β-aminoethylether)-N,N,N'N'-tetraacetic acid. Fura 2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid. HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid. Quin 2, 2-{[2-bis(carboxymethyl)-amino-5-methyphenoxy]-mehtyl]}-6-methoxy-8-bis-(carboxymethyl)-aminoquinoline. R.T., room temperature.

laboratories has been to investigate the effects of CsA on the metabolism of gingival fibroblasts *in vitro*.

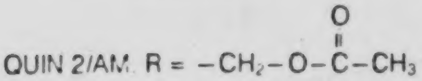
We proposed that if CsA enhanced fibroblast proliferation, it might be responsible for the increases of connective tissue production in CsA-induced gingival hyperplasia (CsA-GH). Another possible mechanism of CsA-GH could involve the inhibition of phagocytosis, which might be physiologically important for fibroblasts *in vivo* (Woolley, 1984). To assess whether CsA acts via a similar mechanism in the fibroblast membrane as the calcium channel blocker nifedipine does in myocardial cell (Antman et al., 1980), the $[Ca^{2+}]i$ (intracellular free calcium ion concentration) can be measured with calcium ion fluorescent chelators Quin 2 or Fura 2.

Quin 2, a tetracarboxylic acid, binds Ca^{2+} with 1:1 stoichiometry. The acetoxymethyl ester Quin 2/AM, which can not bind to Ca^{2+}, can easily diffuse through the cell membrane and then be hydrolysed into Quin 2 by esterase in the cytosol (Fig.2.1). It is thus trapped inside the cytosol but not bound to membranes nor sequestered inside organelles (Tsien, 1980; Tsien et al., 1982). The Quin 2-Ca^{2+} complex has a 5-fold greater fluorescence than Quin 2 or Quin 2/AM (Tsien et al., 1982).

Similarly, the new calcium fluorescence dye Fura 2/AM, the acetoxylmethyl ester form of Fura 2, permeates the cell membrane and is trapped inside the cytoplasm as Fura 2

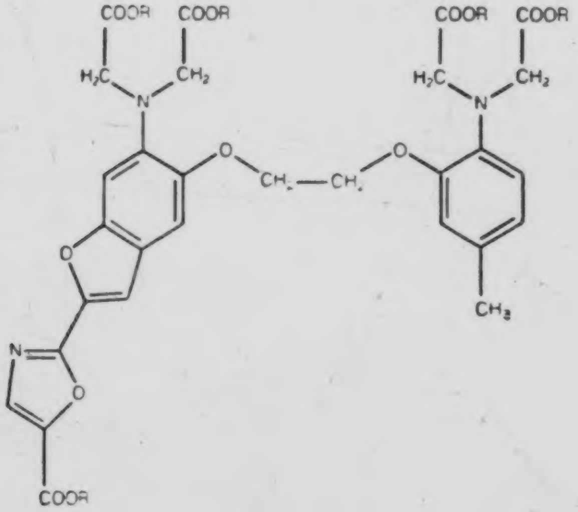


QUIN 2: $R = K^+$

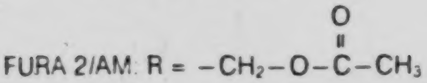


QUIN 2/AM: $R = -CH_2-O-\overset{O}{\overset{\|}{C}}-CH_3$

Figure 2.1 Chemical structures of Quin 2/AM and Quin 2.



FURA 2: $R = Na^+$



FURA 2/AM: $R = -CH_2-O-\overset{O}{\overset{\|}{C}}-CH_3$

Figure 2.2 Chemical structures of Fura 2/AM and Fura 2.

(Grynkiewicz et al., 1985; Tsien et al., 1985). The chemical structures of Fura 2/AM and Fura 2 are shown in Figure 2.2 (Grynkiewicz et al., 1985; Tsien et al., 1985).

The advantages of Fura 2 over Quin 2 are higher fluorescence and quantum yield, lower affinity but higher selectivity for Ca^{2+} over other divalent cation, and the excitation spectrum of Fura 2 shifting to a longer wavelength on binding Ca^{2+} (Grynkiewicz et al., 1985; Tsien et al., 1985). However, the leakage problem for Fura 2 is more severe than that for Quin 2 (Malgaroli et al., 1987).

In the present studies, we investigated the direct effects of CsA on the growth of gingival fibroblasts, on the ability to phagocytose and on $[Ca^{2+}]i$.

2.2. MATERIALS AND METHODS

2.2.1. Materials.

Dulbecco's modified Eagle medium (DMEM) powder, sterilized fetal calf serum (FCS) were from GIBCO Laboratories, Life Technologies Inc., Grand Island, NY., USA. The disposable, sterilized tissue culture supplies (e.g. 75 cm^2 cell culture flasks, flat bottom 24-well or 6-well plates, petri dishes, 15 mL or 50 mL centrifuge polystyrene tubes, cryotubes, 10 mL glass pipettes) were from Corning (NY.,USA), Linbro (Flow Laboratories Inc., McLean, Virginia, USA), Falcon

(Becton Dickinson and Corp., CA., USA), Fisher Scientific (NJ., USA), Nunc (Denmark) and KIMBLE (Ohio, USA). Sterile Millex-GS^{TM} and Sterivex-GS^{TM} (0.22 µm) filters were from Millipore Products division, Bedford, MA, USA. Dry cyclosporine A was a gift from Sandoz Ltd., Basel, Switzerland. Trypsin (1:250) was from DIFCO Laboratories, Michigan, USA. Penicillin-G, gentamycin sulfate, fungizone, Quin 2/AM, Fura 2/AM, A23187, and EGTA were from Sigma Chemical Corp., St. Louis, MO., USA. Polystyrene monodisperse latex (50.87 µm in diameter, S.D.=0.11) was from Polysciences Inc., Warrington, PA, USA. Triton X-100 (electrophoresis purity reagent) was from Bio-Rad Laboratories, Richmond, CA., USA. Other chemicals were from Fisher Scientific.

The medium (DMEM-0) was made from DMEM powder supplemented with $NaHCO_3$ (3.7 g/L), HEPES (4.7 g/mL), penicillin-G (100 mg/L), gentamycin sulfate (50 mg/L), and fungizone (20 mg/L), and passed through a Sterivex-GS^{TM} filter (0.22 µm) under sterile conditions. The DMEM-10 was made from DMEM-0 with 10% (v/v) FCS and used as growth medium. The stock CsA solution was made in 95% ethanol. The final concentration of ethanol in the growth medium was always $\leq$ 0.1%, a level which has no effect on the proliferation or viability of gingival fibroblasts (A. Douglas and P. G. Scott, unpublished data). Phosphate buffered saline (PBS, pH 7.4) contained 137 mM NaCl, 3 mM KCl, 4 mM $Na_2HPO_4.7H_2O$ and 15 mM KH_2PO_4 and was sterilized by autoclave. The physiological saline (PS solution, pH 7.4) contained 145 mM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4.7H_2O$, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 mM ß-(D)(+)-Glucose and 5 mM HEPES and was sterilized by passing through a Sterivex-GS™ filter (0.22 µm). Stock EGTA solution was 100 mM in Tris-HCl buffer (pH 8.5). Triton X-100 was diluted to 5 or 10% (v/v) with distilled water. Stock trypsin solution was prepared at a concentration of 2.5% (w/v) in PBS, passed through a Millex-GS™ filter (0.22 µm) under sterile condition and stored at -20°C. This was thawed and diluted with PBS to give a working solution at 0.25% (w/v).

2.2.2 Gingival fibroblasts: primary explantation, storage, passage and culture.

The methods were derived from Freshney (1983) and Nakano and Scott (1985). Human gingival tissue removed during periodontal surgery was washed several times with DMEM-10 and sliced into small pieces about 1 mm^3. The tissue pieces were rinsed with 20 mL of DMEM-10 five times and recovered by low speed (1000 rpm, R.T.) centrifugation (Model HN-S centrifuge, from International Equipment Company, MASS., USA). Three or four pieces of the tissue were placed in a petri dish and immobilized with a glass cover slid with a drop of sterile grease. DMEM-10 (3 mL/dish) was added and the dishes were placed into a cell culture incubator for four weeks. The cell culture incubator (Forma Scientific or National CO_2 incubator) was always set at 37°C, 95% air, 5% CO_2, 1 atmosphere and 95%

humidity. The DMEM-10 was changed every 7 days for the first two weeks' culture and every 3 to 4 days at later stages.

When the fibroblasts were confluent they were harvested with 0.25% trypsin solution at R.T. Addition of 10 mL DMEM-10 within 5 min was used to terminate the action of trypsin and the cells were recovered by centrifugation (2500 rpm, 10 min, at R.T.). The cells were passaged into 75 cm^2 flasks (labelled "P1") at about $5x10^5$ cells/flask and grown in DMEM-10 for 2 weeks. The fibroblasts were harvested again and then resuspended in fresh DMEM-10 supplemented with 10%(v/v) DMSO (dimethyl sulfoxide) at a density of 1 to $5x10^6$ cells/mL. The cell suspension was transferred to Nunc cryotubes (1.0 to 1.5 mL/vial) which were put in a -50°C freezer overnight. The tubes were then transferred into liquid nitrogen for long term storage. Cell strains were obtained from hyperplastic gingiva of organ transplant patients receiving CsA (responder cell strains), non-hyperplastic gingiva in the CsA-treated patients with hyperplasia at other locations or CsA-treated patient without hyperplasia at all (non-responder cell strain), and from clinically non-inflamed tissue from individuals not receiving CsA therapy (normal cell strains) (Table 2.1).

Table 2.1 Human gingival fibroblast cell strains used for *in vitro* study.

Classification	Name of the cell strains
Responder cell strain	HUGE-7B
	CSA-8
	CSA-11
	CSA-12
Non-responder cell strain	HUGE-7A
	HUGE-6
Normal cell strains	N1106
	N1806
	N606B
	HUGE-5
	HUGE-12
	HUGE-15

HUGE-7A and HUGE-7B were from the same CsA-treated patient.

In order to assess effects of CsA on fibroblast proliferation, a suspension (1 mL) containing about $0.5x10^4$ cells/mL was added to each well of a 24-well plate. Medium was replaced at day 1 and every 2 to 3 days during the culture period, at which time cells were harvested from two wells. The samples were centrifuged briefly (Microfuge, Beckman) and the supernatants were discarded. Each sample was resuspended in 1 mL PBS and mixed with 19 mL Isotone™ solution (Fisher) and counted eight times with a Coulter counter (model ZM).

In order to study the phagocytic activity of fibroblasts, cells were cultured with polystyrene latex beads in DMEM-10, as described above. The medium was prepared by adding 18 drops of polystyrene latex beads to 30 mL DMEM-10. After thorough mixing and standing at R.T. for 30 min, the tubes were centrifuged at 2500 rpm for 20 min at R.T. The supernatant was discarded. The beads were washed twice more with 30 mL DMEM-10. The final suspension contained 3.3×10^6 beads/mL.

When the fibroblasts grown in DMEM-10 in 6-well plates were confluent, the medium was replaced with fresh DMEM-10 containing CsA 0, 200, 400 and 800 ng/mL. After 24 hours' culture, 0.5 mL of the bead suspension was added into each well and incubation continued under the same conditions for 2 h. or 24 h. The cells were harvested as usual and suspended in 1 mL PBS per well for counting the number of beads and total number of cells with a haemocytometer under a Diavert inverted light microscope.

2.2.3 Measurement of intracellular free calcium ion concentration.

The methods were modified from Tsien et al.(1982), Rink and Pozzan (1985) and Malgaroli et al. (1985). The stock solutions of the fluorescent dyes were in DMSO at 50 mM for Quin 2/AM and 5 mM for Fura 2/AM and stored at -20°C in a desiccator in the dark. The final concentrations of DMSO in the cell suspensions were always $\leq 0.5\%$ (v/v) (Tsien et al., 1982).

When the fibroblasts had grown to confluence (in about 2 weeks) in DMEM-10, the cells were harvested as above and suspended in 1 mL of PS solution at a density of 1×10^7 cells/mL with 50 µM Quin 2/AM (final concentration) or 5 to 10 µM Fura 2/AM (final concentrations). Samples were incubated at 37°C in a water bath for 20 min, then diluted 10-fold with PS solution and incubated for an additional 45 to 60 min. The samples were centrifuged and the supernatants were discarded. The cell pellets were resuspended in 10 mL PS solution and stored at R.T. in the dark.

For Quin 2 fluorescence measurement, the excitation wavelength of the Perkin-Elmer LS-5 fluorometer spectrophotometer was set to 339 nm (slit width = 10 nm, the nominal bandpass) and the emission wavelength to 492 nm (slit width = 20 nm). For Fura 2, excitation was at 340 nm and emission at 510 nm. The cells were resuspended in the same volume of PS solution immediately before measurement. The fresh cell suspension (0.1 to 0.2 mL) was pipetted into 2.4 to 2.3 mL PS solution in a 1x1 cm^2 quartz quality cuvette. The fluorescence (F. value) was measured at 37°C with/without addition of A23187 (100 ng/mL) and/or CsA (800 ng/mL). The calibration of $[Ca^{2+}]i$ was done by adding 5 to 10 µL of the detergent Triton X-100 (0.22%, v/v, final concentration) to rupture the cell membrane to get the maximum fluorescence value (F_{max}), followed by adding 100 µL of EGTA solution (3.85 mM final concentration) to get minimum fluorescence (F_{min}). The actual $[Ca^{2+}]i$ value was calculated using the following equation (Tsien et al., 1982; Grynkiewicz et al., 1985):

$$[Ca^{2+}]i = K_d \times \frac{F. - F_{min}}{F_{max} - F.}$$

Where the dissociation constants (K_d) of the dyes with Ca^{2+} were determined in the presence of 120 to 130 mM K^+, 20 mM Na^+, 1 mM Ca^{2+} and 1 mM free Mg^{2+} (pH 7.05) for Quin 2 (K_d=115 nM) (Tsien et al., 1982); or in presence of 115 mM KCl, 20 mM NaCl, 10 mM K-MOPS and 1 mM free Mg^{2+} (pH 7.05, 37°C) for Fura 2 (K_d=224 nM) (Grynkiewicz et al, 1985).

2.2.4 Statistics.

For cell proliferation rates, growth curves were fitted to the data (number of cells/well for each day counted) by third order polynomial regressions (Sigmaplot™ software) with the coefficient values (R. values) calculated for each group ([CsA] 0, 200, 400, 800 ng/mL) and the "wholeplot" (average of the above four plots in each cell strain). For the rest of the experimental data in this paper, standard deviations (S.D.) were calculated from the raw data and comparison of means was done by Student's t-test (t-test).

2.3 RESULTS

2.3.1 Effect of cyclosporine A on fibroblast proliferation rate.

Sixteen experiments on the effects of CsA on fibroblast growth were carried out using seven different cell strains (N606B, N1106, HUGE-5, CSA-8, CSA-11, HUGE-7b and HUGE-7a, passage 3 to 10). In all of these polynomial plots (including the "whole plot", see below), five of them have good correlation with the R values from 0.81 to 0.89 and only one has fair correlation with R = 0.75, while the rest of the plots are all highly correlated to each other with R values from 0.92 to 0.99. Only partial data are given here, i.e., the growth curves for two cell strains are shown in figures 2.3.a and 2.3.b. Thus, from this study, we concluded that CsA in the culture medium at concentrations of 200 to 800 ng/mL had no significant effect on the rate of growth of gingival fibroblasts.

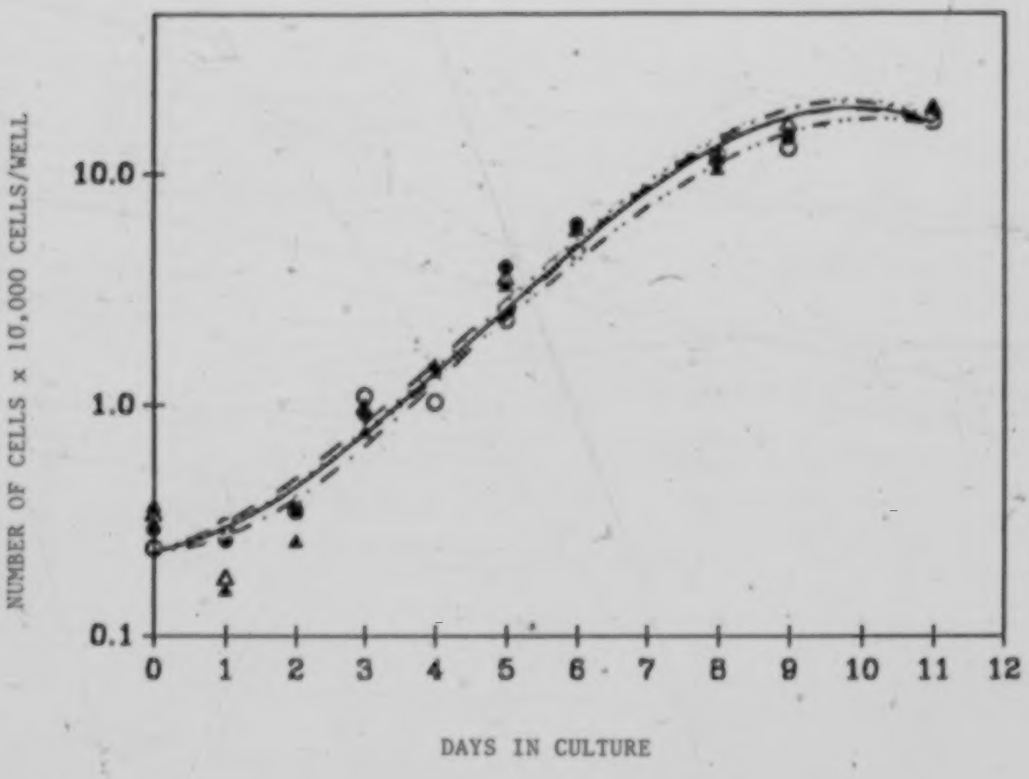


Figure 2.3.a Effect of cyclosporine A on fibroblast (N1106, passage 5) proliferation. Polynomial regressions for each CsA concentration used were: (o-··-··-o-··-··-o) CsA 0 ng/mL (R=0.993); (●---●---●) CsA 200 ng/mL (R=0.992); (▲····▲····▲) CsA 400 ng/mL (R=0.988); (▲-·-·-▲-·-·-▲). CsA 800 ng/mL (R=0.982). (———) "Whole plot" (R = 0.987).

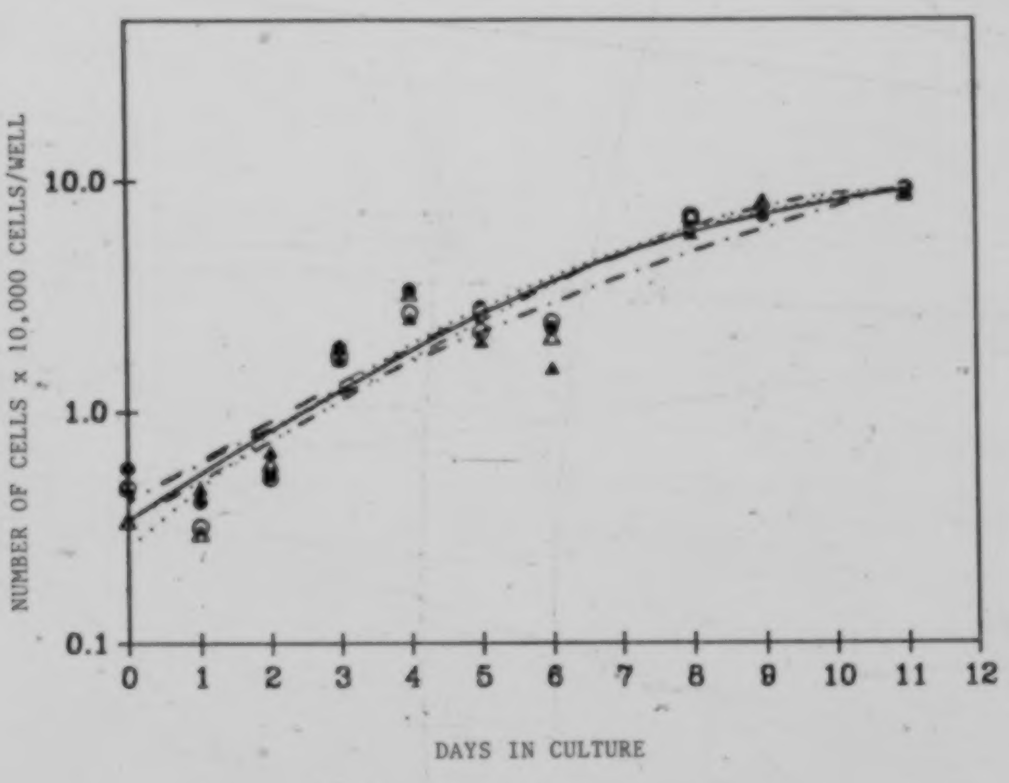


Figure 2.3.b Effect of cyclosporine A on fibroblast (CSA-11, passage 5) proliferation. Polynomial regressions for each CsA concentration used were: (o-••-••-o-••-••-o) CsA 0 ng/mL (R=0.962); (●---●---●) CsA 200 ng/mL (R=0.946); (△····▲····▲) CsA 400 ng/mL (R=0.959); (▲-•-•-▲-•-•-▲) CsA 800 ng/mL (R=0.954). (————) "whole plot" (R = 0.952).

2.3.2 Effect of cyclosporine A on phagocytosis.

The latex beads phagocytosed by fibroblasts were easily visible under the light microscope as tiny black rings. The number of beads inside the cells in the same culture varied from 0 to 10 beads. Cell membranes remained intact as revealed by staining with 0.04% trypan blue.

Table 2.2 Effect of cyclosporine A on the phagocytosis of latex beads.

a. Incubation for 2 hours.

Cell strain (passage)	[CsA] (ng/mL)	Percentage of cells with beads	No. of beads per cell with beads	No. of beads per cell
N1106 (P5)	0	27.7±6.3	2.2±0.4	0.6±0.0
	200	28.3±5.6 (p=0.76)	2.3±0.2 (p=0.49)	0.6±0.1 (p=0.20)
	400	26.2±4.4 (p=0.56)	2.2±0.1 (p=0.82)	0.6±0.2 (p=0.89)
	800	29.5±2.8 (p=0.87)	2.1±0.1 (p=0.70)	0.6±0.0 (p=0.48)

(Table 2.2.a contd.)

Cell strain (passage)	[CsA] (ng/mL)	Percentage of cells with beads	No. of beads per cell with beads	No. of beads per cell
CSA-11 (P5)	0	38.6±4.4	2.2±0.1	0.9±0.2
	200	39.0±2.6 (p=0.86)	2.1±0.2 (p=0.56)	0.8±0.1 (p=0.82)
	400	30.2±2.9 (p=0.92)	1.9±0.2 (p=0.03*)	0.7±0.1 (p=0.28)
	800	39.4±6.0 (p=0.08)	2.3±0.5 (p=0.59)	0.7±0.1 (p=0.26)

Table 2.2 Effect of cyclosporine A on the phagocytosis of latex beads.

b. Incubation for 24 hours.

Cell Strain (passage)	[CsA] (ng/mL)	Percentage of cells with beads	No. of beads per cell with beads	No. of beads per cell
N1106 (P5)	0	36.5±1.8	2.6±0.4	0.8±0.0
	200	45.5±4.2 (p=0.11)	2.6±0.3 (p=1.0)	1.2±0.2 (p=0.19)
	400	34.8±4.1 (p=0.65)	2.6±1.0 (p=1.0)	0.9±0.2 (p=0.80)
	800	34.6±7.0 (p=0.75)	2.6±0.3 (p=0.88)	1.0±0.0 ($p=0.04^*$)

(Table 2.2.b contd.)

Cell strain (passage)	[CsA] (ng/mL)	Percentage of cells with beads	No. of beads per cell with beads	No. of beads per cell
CSA-11 (P5)	0	32.8±3.5	2.4±0.3	0.8±0.0
	200	44.3±0.2 ($p=0.05^*$)	2.7±0.2 ($p=0.35$)	1.2±0.0 ($p<0.01^*$)
	400	49.6±4.7 ($p=0.06$)	2.6±0.1 ($p=0.47$)	1.3±0.2 ($p=0.06$)
	800	48.7±0.8 ($p=0.03^*$)	2.3±0.1 ($p=0.70$)	1.1±0.1 ($p=0.04^*$)

Results shown in Table 2.2.a and 2.2.b are the averages for triplicate wells and are expressed as Mean ± 1 S.D. Statistical difference* (Student's t-test) between results at [CsA] given and 0 ng/mL is accepted at $p\leq0.05$.

After 2 and 24 hours' incubation, the cell suspensions (in PBS) at a density of about 7.5×10^5 cells/mL/well were counted under the microscope as described in above. At the later time, only slightly increases in phagocytosis were seen.

For a normal cell strain (N1106, P5) there were no statistically significant differences in the number of beads phagocytosed by the fibroblasts in the presence or absence of CsA (200 to 800 ng/mL) after 2 hours' incubation (Table 2.2.a). Only a slight increase in number of beads per cell at CsA 800 ng/mL after 24 hours' incubation (Table 2.2.b) was seen. For CsA-GH cell strain (CSA-11, P5), only the number of beads per cell with beads at one group (CsA 400 ng/mL) was slightly decreased after 2 hour's incubation, while at later time CsA (at 200 ng/mL and 800 ng/mL) slightly enhanced the number latex of beads phagocytosed by the fibroblasts.

2.3.3 Effect of cyclosporine A on intracellular free calcium ion concentrations in the presence and absence of the calcium ionophore A23187.

It was found to be important to grow the fibroblasts for a sufficient time and to confluence in order to get consistent results in the experiment since proliferating fibroblasts tend to have a higher [Ca^{2+}]i than quiescent cells (≤ 200 nM) (X.F. Fan and P.G. Scott, unpublished data). If the fibroblasts were cultured for 14 days to confluence the resting [Ca^{2+}]i was about 150 nM (measured with either Quin 2 or Fura 2).

CsA *per se* did not affect the [Ca^{2+}]i at 800 ng/mL (final concentration) when it was directly added into the fluorescent dye loaded cell suspensions in a cuvette and studied within 15 min (Table 2.3) or incubated with the cells for periods from 2 hours to 16 hours at 37°C prior to loading with the fluorescent indicator (Tables 2.4 and 2.3). The calcium ionophore A23187 at 100 ng/mL (but not at 40 ng/mL, data not shown) increased $[Ca^{2+}]i$ about 2-fold within 30 seconds and maintained it at that level for at least 20 min (Table 2.4). Pretreatment of the cells with CsA (800 ng/mL) for 2 hours did not inhibit the $[Ca^{2+}]i$ increases stimulated by A23187 (Table 2.4).

Table 2.3 Direct effect of cyclosporine A (800 ng/mL) on intracellular free calcium ion concentrations ($[Ca^{2+}]i$).

Cell strain (passage No.)	$[Ca^{2+}]i$ (nM) ± 1 S.D. Before CsA added	$[Ca^{2+}]i$ (nM) ± 1 S.D. After CsA added	P values in t-test
N606B (P10)	154.1 ± 15.5	138.5 ± 19.0	0.33
CSA-8 (P5)	151.9 ± 29.0	232.3 ± 46.9	0.07
CSA-8 (P6)*	141.6 ± 46.8	134.9 ± 36.9	0.86

This experiment was done with Quin 2. *The cells were preincubated with CsA (800 ng/mL) for 16 hours before measurement. Non-statistical difference is accepted at $p \geq 0.05$

Table 2.4 Effect of cyclosporine A on fibroblast (N1106, passage 6) intracellular free calcium ion concentrations ($[Ca^{2+}]i$) in the absence and presence of A23187.

A23187 (100 ng/mL)	$[Ca^{2+}]i$ (nM) ± 1 S.D. No CsA	 CsA = 800 ng/mL*	(P value in t-test)
Before adding	148.6± 2.8	141.9±20.3	(0.69)
After adding	315.7±23.8	340.6±37.3	(0.51)
(P value in t-test)	(0.01)	(0.02)	

This experiment was done with Fura 2. *The sample was pretreated with CsA for 2 hours before measurement of $[Ca^{2+}]i$. Significant difference is accepted at $p \leq 0.05$.

At the end of the experiment, about 85 to 90% of the Quin 2 or Fura 2 loaded fibroblasts were viable as determined by exclusion of trypan blue. All the fluorescence measurements were made within 2 hours after loading with Quin 2 or Fura 2. Otherwise, inconsistent results were obtained.

2.4 DISCUSSION

One of the direct hypotheses to explain CsA-GH was that CsA might act as a mitogen for fibroblasts and therefore enhance the production of connective tissue. If the synthesis and catabolism of collagen are not balanced, i.e., either collagen synthesis is enhanced or collagen catabolism is depressed, or both, there would be a net increase in collagen content in the tissue.

The blood CsA levels in patients vary greatly with the same dose and it was recommended to maintain the CsA concentrations in serum within the range of 100 to 300 ng/mL which normally prevents rejection and minimizes CsA-associated toxicity (Bowers and Canafax, 1984). The upper level of CsA concentration range (200 to 800 ng/mL) used in our experiments was higher than the recommended CsA levels in the blood, but there was no toxicity apparent in the fibroblast cultures. Previously, it was found that high concentrations of CsA (1 and 5 μg/mL) in the culture medium were toxic to the fibroblasts after 1 week exposure, while CSA at 25 μg/mL was cytotoxic by day 2 (C.M. Dodd and P.G. Scott, unpublished data).

Because of the high hydrophobicity of CsA, nonspecific adsorption to the wall of the plastic cell-culture dishes is a potential problem. This did appear to occur to some extent and was found to be proportional to the contact area (X.F.

Fan and P.G. Scott, unpublished data). However, the loss was less than 10% of the total CsA added in the culture medium after culture in a 6-well-plate with or without cells for 24 hours. More than 95% of this "loss" of CsA was associated with the cells if there was a confluent layer of cells on the bottom of the well (see Chapter III). Therefore, the actual non-specific adsorption of CsA to the plastic wall was negligible.

Our negative results on the effect of CsA on proliferation of fibroblasts are in conflict with Zebrowski et al. (1986) who reported an increase, as measured by DNA contents of the culture. Tipton and Dabbous (1986) reported no effect, a conclusion supported by the present results. The reason for the different results is not clear.

The ability of fibroblasts to phagocytose may be an important biological function of the cell since this stimulates fibroblasts to produce collagenase (Werb and Reynolds, 1974). Fibroblasts also degrade phagocytosed collagen fibril fragments and this may be physiologically important (Sodek and Overall, 1988). If CsA inhibited phagocytosis in vitro, it could be suspected that the fibroblasts' capacity to degrade collagen fibrils would be affected by the drug and this could contribute to the mechanism of CsA-GH. However, our results showed that CsA slightly enhanced fibroblast's ability to phagocytose latex beads after 24 hours' incubation. This effect was more obvious in a CsA-GH cell strain than in a normal cell strain. It is not clear what is the physiological or pathological significance of this effect of CsA on fibroblasts, or wether it is related to the mechanism of CsA-GH.

Intracellular calcium ion is an important second messenger (Carafoli, 1987). It has been reported that pretreatment of human T lymphocytes or mouse spleen cells with CsA for 30 min significantly inhibited the phytohemagglutinin (PHA)- or concanavalin A (ConA)-induced Ca^{2+} influx (Gelfand et al., 1987; Chatenoud et al., 1988). Our results showed that CsA did not cause Ca^{2+} influx in fibroblasts. We also found that CsA did not inhibit the calcium influx initiated by the calcium ionophore A23187. It may not strictly be correct to compare the effect of CsA on mitogen-stimulated Ca^{++} influx in lymphocytes with A23187-induced Ca^{2+} influx in fibroblasts. However, we were not successful in attempting to detect Ca^{2+} changes in response to ConA treatment in the suspended fibroblasts. Pretreating the fibroblasts with ConA created a problem in trypsinizing the cells off the culture flask (X.F. Fan and P.G. Scott, unpublished data). If we had been able to study attached fibroblasts, we could have compared our results with those in lymphocytes.

Although it is clear that Ca^{2+} influx is a critical step in early events of T lymphocyte activation in response to mitogens such as ConA and PHA (Whitney and Sutherland, 1972; Tsien et al., 1982), how stimulation of fibroblast proliferation, phagocytosis, synthesis and secretion of collagen and collagenase might relate to changes in $[Ca^{2+}]i$ is unknown. Recently, the report of stimulation by A23187 (40 ng/mL) of rabbit synovial fibroblasts to produce collagenase was the first evidence relating Ca^{2+} influx to collagenase production (Unemori and Werb, 1988). However, we did not detect a Ca^{2+} influx at 40 ng/mL A23187. Furthermore, it is interesting to note that the calcium channel blocker nifedipine can cause the similar side-effect to CsA and phenytoin (Butler et al., 1987), and that phenytoin inhibited the voltage-gated Ca^{2+} influx in the PC12 pheochromocytoma cell line (Messing et al., 1985). These observations all suggest that Ca^{2+} is a very important second messenger in the regulation of collagen metabolism.

The negative results shown in this paper strongly suggest that CsA may not act on the fibroblast membrane. However, more direct evidence could be obtained if the effects of CsA on the cell membrane potential and/or membrane fluidity could be measured. Nevertheless, the collagen content increase in the drug-associated gingival hyperplasia leads to further investigation of CsA effects in the metabolism of collagen by fibroblasts (see Chapter 4 for details).

2.5 BIBLIOGRAPHY:

ANTMAN, E.M., STONE, P.H., MULLER, J.E., BRAUNWALD, E. Calcium channel blocking agents in treatment of cardiovascular disorders. Part I: Basic and clinical electrophysiologic effects (Reviews). Ann. Internal Med. 93:875-885, 1980.

BENNETT, W.M., NORMAN, D.J. Action and toxicity of cyclosporine. Ann. Rev. Med. 37:215-224, 1986

BOWERS, L. CANAFAX, D.M. Cyclosporine: Experience with therapeutic monitoring (review). Therapeutic Drug Monitoring. 6:142-147, 1984.

BUTLER, .RT., KALKWARF, K.L., KALDAHL, W.B. Drug-induced gingival hyperplasia: phenytoin, cyclosporine, and nifedipine. J. Am. Dent. Assoc. 114:56-60, 1987.

CARAFOLI, E. Intracellular calcium homeostasis. Ann. Rev. Biochem. 56:395-433, 1987.

CHATENOUD, L., DUGAS, B., DAMAIS, C., BACH, J-F. In vitro cyclosporine A treatment inhibits spleen cell mitogen-triggered free [Ca^{2+}] increase. Transp. Proceedings. 20, Suppl. 2:170-172, 1988.

DALEY, T.D., WYSOCKI, G.P., DAY, C. Clinical and pharmacologic correlations in cyclosporine-induced gingival hyperplasia. Oral Surg. Oral Med. Oral Path. 62:417-421, 1986.

FRESHNEY, R.I. Culture of animal cells: A manual of basic technique. Alan R. Liss, Inc., NY., 1983.

GELFAND, E.W., CHEUNG, R.K. MILLS, G.B. The cyclosporins inhibit lymphocyte activation at more than one site. J. Immunol. 138:1115-1120, 1987.

GRYNKIEWICZ, G., POENIE, M., TSIEN, R.Y. A new generation of of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260:3440-3450, 1985.

LUCAS, R.M., HOWELL, L.P., WALL, B.A. Nifedipine-induced gingival hyperplasia: A histochemical and ultrastructural study. J. Periodontol. 56:211-215, 1985.

MALGAROLI, A., MILANI, D., MELDOLESI, J., POZZAN, T. Fura-2 measurement of cytosolic free Ca^{2+} in monolayers and suspensions of various types of animal cells. J. Cell Biol. 105:2145-2155, 1987.

MESSING, R.O., CARPENTER, C.L. GREENBERG, D.A. Mechanism of calcium channel inhibition by phenytoin: Comparison with classical calcium channel antagonists. J. Pharmacol. and Exp. Therapeutics. 235:407-441, 1985.

NAKANO, T., SCOTT, P.G. Purification and characterization of a gelatinase produced by fibroblasts from human gingiva. Biochem. Cell Biol. 64:387-393, 1986.

RINK, T.J., POZZAN, T. Using Quin 2 in cell suspensions. Cell Calcium. 6:133-144, 1985.

SODEK, J., OVERALL, C.M. Matrix degradation in hard and soft connective tissues. In: The Biological Mechanisms of Tooth Eruption and Root Resorption, Z. Davidovitch editor. EBSCO Media, Birmingham, 1988. pp. 303-311.

TIPTON, D.A, DABBOUS, M.K. Heterogeneity of gingival fibroblast response to cyclosporine. J. Dent. Res. 65 (Special issue):331, 1986.

TSIEN, R.Y., POZZAN, T., RINK, T.J. Calcium homeostasis in intact lymphocytes: Cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. J. Cell Biol. 94:325-334, 1982.

TSIEN, R.Y., POZZAN, T., RINK, T.J. T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. Nature. 295:68-71, 1982.

TSIEN, R.Y., RINK, T.J., POENIE, M. Measuring and manipulating cytosolic free-Ca^{++} in individual small cells using fluorescence microscopy with dual excitation wavelengths. Cell Calcium. 6:145-157, 1985.

UNEMORI, E.N., WERB, Z. Collagenase expression and endogenous activation in rabbit synovial fibroblasts stimulated by the calcium ionorphore A23187. J. Biol. Chem. 263: 16252-16259, 1988.

WERB, Z., REYNOLDS, J.J. Stimulation by endocytosis of the secretion of collagenase and neutral proteinase from rabbit synovial fibroblasts. J. Exp. Méd. 140:1482-1497, 1974.

WHITNEY, R.B. SUTHERLAND, R.M. Enhanced uptake of calcium by transforming lymphocytes. 5:137-147, 1972

WOOLLEY, D.E. Mammalian collagenase. In: Extracellular Matrix

Biochemistry. K. A. Piez and A. H. Reddi editors. Elsevier, NY., Amsterdam, Oxford, 1984. pp.119-157.

WYSOCKI, G.P., GRETZINGER, H.A., LAUPACIS, A., ULAN, R.A. STILLER, C.R. Fibrous hyperplasia of gingiva: A side effect of cyclosporin A therapy. Oral Surg. Oral Med. Oral Path. 55:274-278, 1983.

ZEBROWSKI, E.J., SINGER, D.L., BRUNKA, J.R. Cyclosporin A, nifedipine and phenytoin. comparative effects on gingival fibroblast metabolism. J. Dent. Res. 65 (Special issue):331, 1986.

CHAPTER III

KINETICS OF UPTAKE AND DISTRIBUTION OF CYCLOSPORINE A IN GINGIVAL FIBROBLASTS IN VITRO

3.1 INTRODUCTION

During long term medication with cyclosporine A (CsA) such as is used to prevent transplant rejection, CsA-associated gingival hyperplasia can develop, as discussed in detail elsewhere (Daley et al., 1984 and Chapter I). We had been using human gingival fibroblasts as a model in *in vitro* studies of CsA-induced gingival hyperplasia (CsA-GH). Previously, we found that CsA had no effect on the proliferation of gingival fibroblasts, phagocytosis and intracellular free calcium ion concentration (Chapter II). Before studying the effect of CsA on collagen catabolism (see Chapter IV), it was thought necessary to determine whether the drug can enter fibroblasts. If so, it would be useful to know how and where it goes inside the cell.

There is no report in the literature on CsA uptake and binding in fibroblasts. However, many studies on CsA uptake have been done with human and murine lymphocytes and lymphoma or thymoma. LeGrue et al (1983) found that CsA bound to human lymphocytes with dissociation constants (K_d) in the range of 3 to 6×10^{-7} M for T cells and 5 to 8×10^{-7} M for B cells, respectively. The B cells also have a high affinity cytosolic site with a $K_d = 2x10^{-9}$ M (LeGrue et al., 1983). Most of CsA (70 to 80%) taken up by murine thymoma was associated with two cytosolic components. One of these was saturable at low drug concentrations (30 to 1000 ng/mL) and the other was non-saturable at higher drug concentrations (≥1000 ng/mL). Merker and Handschumacher (1984) and Fabre et al. (1986) also reported similar results on human lymphoma and stressed that the drug was rapidly accumulated inside the cells and reached a near steady-state within 1 to 3 min. The apparent M.W. for the CsA-binding molecule in the cytosol of lymphocytes cytosol was 15,000 to 20,000 dalton (Merker and Handschumacher, 1984; Fabre et al, 1986). CsA receptors were identified in in vitro studies as a new cytosolic protein "cyclophilin" (Handschumacher, 1984) and a well known calcium regulated protein calmodulin (Colombani et al. 1985). The dissociation constants for cyclophilin are in the range of 10^{-8} M (Dalgarno et al., 1986; Hess et al., 1988) to 10^{-7} M (Handschumacher et al., 1984) and for calmodulin 10^{-7} M (Colombani et al., 1985; Hess et al., 1988) to 10^{-6} M (LeGrue et al, 1986).

The purposes of the present study were (a) to measure the kinetics of uptake and distribution of CsA in human gingival fibroblasts; (b) to investigate the effect of ouabain, an inhibitor of Na^+/K^+-dependant ATPase, on CsA uptake by the fibroblasts. However, the CsA-binding molecule in the cytosol is not identified in this study.

3.2 MATERIALS AND METHODS

Both non-radioactive CsA and [^{3}H]-CsA (450 µCi/mL, 50.1 µg/mL) were gifts from Sandoz Ltd., Basel, Switzerland. Human gingival cell strains used in the experiments were the same as before (see chapter II for details). The ultracentrifuge tubes (polyallomer) were from Beckman. NCS tissue solublizer (containing toluene and quaternary ammonium hydroxide) was from Amersham, USA. Universal LSC cocktail (SO-X-16, Scinti Verse™ E) was from Fisher scientific company. The cell culture supplies were the same as those used in chapter II.

Fibroblasts were grown in 25 cm^2 flasks or 6-well flat bottom plates in DMEM-10, i.e., Dulbecco's modified Eagle's medium containing 10% fetal calf serum (see chapter II), until confluence. The cells in fresh DMEM-10 were then labelled with tritiated CsA at a concentration of 800 ng/mL with desired specific activities (SA) for 0.5 to 25 hours, or at concentrations of 100 ng/mL to 4000 or 5000 ng/mL for 1 to 2 hours in a CO_2 incubator (Forma Scientific Company) which was set up as before (Chapter II). In one experiment, [CsA] in the medium was up to 10 µg/mL and incubation for 24 h. At the end of culture, the medium in all wells was collected for measuring the total [^{3}H]-CsA left by counting 0.1 or 0.3 mL medium with 5 mL universal LSC cocktail. The results were used as total free [CsA] in Scatchard plots (see below). The cells were then harvested with 0.25% trypsin by the conventional method (chapter II) and washed with PBS three times. The last wash with PBS (1 mL/ sample) was collected, and counted to check for completeness of washing and removal of unbound CsA. The volume (V) of a suspended fibroblast was calculated by using the formula V= $(3.14/6)d^3$ (d is the diameter of a spherical fibroblast in suspension and was determined with a Coulter counter). Fresh PBS (1 mL) was added to the cell pellets which were then frozen (at -20°C) and thawed (at room temperature) three times each for 2 h., respectively to erupture the cells. After this procedure, more than 95% of the cells were stained positively by 0.04% trypan blue and therefore had been ruptured. The cells treated with non-radioactive CsA-containing medium were used as a control for counting total number of cells and radioactive background of the cells; while the wells without cells were treated with radioactive CsA-containing medium to measure the non-specific adsorption of CsA in the plastic tissue culture dishes.

The cytosol and cell residues, including all insoluble debris of the cells were separated by centrifugation (Beckman L8-70 ultracentrifuge, SW Ti 50 rotor) at 10,000xG, 4°C for 2 hours. After the supernatant (called the "1st cytosol fraction") was collected, the cell pellet was resuspended in 1 mL fresh PBS and centrifuged for 30 min as above. These supernatants (called the "2nd cytosol fraction") were collected. All these fractions (last wash with PBS, 1st cytosol fraction, 2nd cytosol fraction and cell residue, each in 1 mL PBS) were mixed with universal LSC cocktail (5 mL/sample) in plastic LSC (liquid scintillation counter) vials and counted in a liquid scintillation counter (Model PW 470, Philips, Holland).

The [F] values (total free ligand concentrations) were calculated from the total [^{3}H]-CsA left in the medium in the end of the cultures. The [F] values were presented as molar concentrations by converting the data from dpm to mole with the theoretical specific activities (S.A.) of CsA added in the medium. The [B] (bound fraction of CsA in the cytosol) was from "1st cytosol fraction". The "2nd cytosol fraction" was so little that it was ignored (see below). The Scatchard plot was made by plotting [B]/[F] versus [B].

To determine whether the transport of CsA across cell membranes involves an active process, confluent fibroblasts in 6-well cell culture plates were incubated with DMEM-10 containing ouabain (1×10^{-8} to 1×10^{-6} M) and [^{3}H]-CsA (800 ng/mL, SA = 125 dpm/ng CsA) in the culture medium for 2 hours. The fractionation and counting were as above.

3.3 RESULTS

3.3.1 Distribution of cyclosporine A in gingival fibroblasts.

After culture of gingival fibroblasts with [^{3}H]-CsA (800 ng/mL, 41.8 dpm/ng CsA) at 37°C for 0.5 h. or 24 h, it was found that 78% (at 0.5 h.) to 88% (at 24 h.) of the total cell-associated CsA was in the two cytosol fractions (Table 3.1.a). The total cell-associated CsA was about 4.2% of the total CsA added in the culture medium (Table 3.1.b).

Table 3.1.a Distributions of cyclosporine A in 1.7×10^6 fibroblasts (N1106, passage 6).

Fraction (in 1 ml/well)	Group A (0.5 h.) (Mean DPM ± S.D.)	Group B (24 h.) (Mean DPM ± S.D.)
1st cytosol	3600±154	4433±263
2nd cytosol	85± 9	92± 11
Cell residue	1044± 35	641± 84
Last wash with PBS	879±109	477±148

The data are average of 10 replicate cultures in $25mm^2$ flasks.

Table 3.1.b Distribution of cyclosporine A in the culture system.

Item	Group A (0.5 h)	Group B (24 h)
(1) Total cell-associated CsA (dpm/1.7×10^6 cells)	5607	5643
(2) Total CsA left in the medium after culture with cell (dpm/flask)	98239	104334
(3) Total CsA left in the medium after incubation without cells (dpm/flask)	91508	103580
(4) Total CsA added into medium (dpm/flask)	133745	133745
(5) percentage of (1) in (4)	4.2%	4.2%
(6) percentage of (2) in (4)	73.5%	78.0%
(7) percentage of (3) in (4)	68.4%	77.5%

From this table it can be seen that the actual non-specific loss of CsA was about 17.8% to 22.9% of the total CsA added into the medium in a 25 cm^2 cell culture flask.

3.3.2 Cyclosporine A uptake with time.

After incubation of CsA (432 ng/mL, 1.53 dpm/ng CsA) with the confluent fibroblasts (CSA-8, passage 5) in 6-well cell culture plates in the CO_2 incubator for 0.5 h. to 25 h., it was found that CsA was rapidly taken up into the cytosol (Figure 3.1).

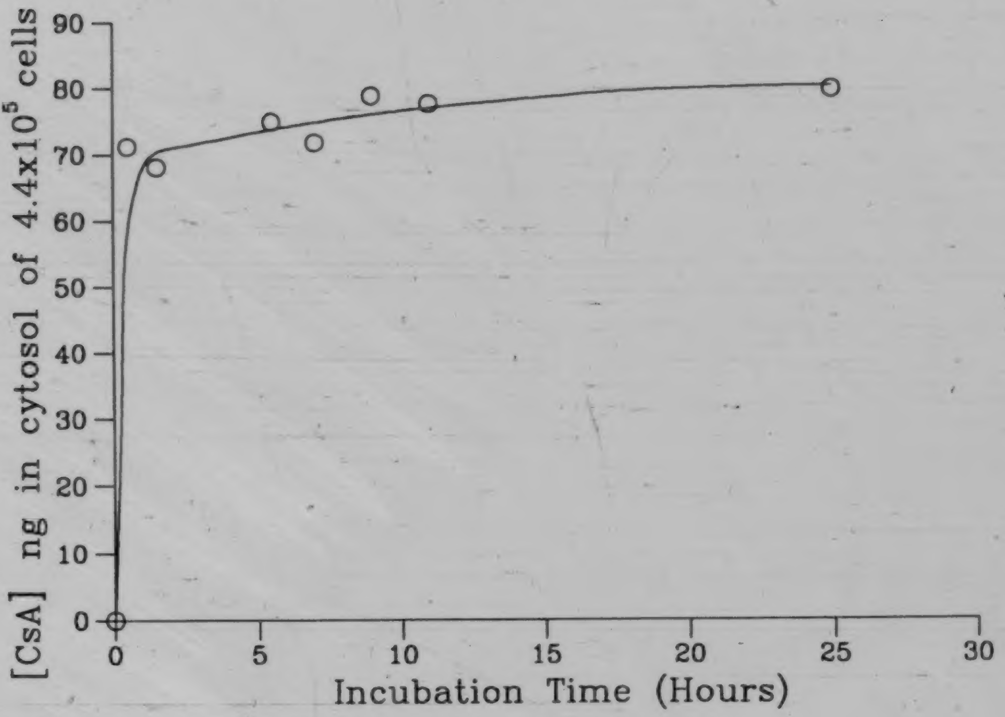


Figure 3.1 Fibroblasts (N1106, passage 5) uptake of cyclosporine A with time. The data are average of duplicate determinations.

3.3.3 Kinetics of cyclosporine A uptake in fibroblasts.

The analysis of the CsA-binding curve in fibroblasts cytosol was based on the assumption that CsA taken up into cytosol was bound to cytosolic molecule(s) since it was highly concentrated. When the concentrations of CsA added in the culture medium were at 100 to 4000 or 5000 ng/mL, the binding curve (Scatchard plot) was non-linear. Four cell strains had been used for the CsA-binding assay. Data for Scatchard plot from one cell strain (HUGE-5) are presented in Table 3.2 and Figure 3.2. The K_d for the low affinity binding site in the Scatchard plots for all these 4 cell strains was close to infinite, which might reflect non-specific adsorption of CsA to the cytosolic protein(s). The uptake associated with the high affinity CsA-binding molecule saturated at 1 µg/mL of CsA in the medium, but the low affinity one(s) did not saturate at concentrations of CsA up to 4, 5 or 10 µg/mL (data not shown for 10 µg/mL).

Fibroblasts from normal cell strain (HUGE-5, passage 5), CsA-GH non-responder cell strains (HUGE-7a and HUGE-6, both in passage 4) and CsA-GH responder cell strains (HUGE-7b, passage 4) behaved in a similar way in the uptake of CsA. After fitting the hyperbolic curve by computer programming to resolve the equations of 2.a or 2.b (see below) for these non-linear Scatchard plots, the dissociation constants (K_d) for the high affinity molecule were calculated (Table 3.3).

The non-specific adsorption to the wells was very little when CsA was incubated in a 6-well plate with a layer of confluent cells (Table 3.4).

Table 3.2 Binding of cyclosporine A by gingival fibroblasts (Normal cell strain: HUGE-5, passage 5).

Medium [CsA] added (ng/mL)	[B] of CsA (ng/1x10^5 cells)	[F] of CsA (x10^{-7} M)/well	[B]/[F] (x10^7)
100	6.2±1.4	0.8±0.1	8.2±2.4
200	9.5±2.0	1.5±0.3	6.7±2.2
400	12.0±2.4	3.4±0.2	3.6±0.7
600	13.9±2.8	5.3±0.1	2.6±0.5
800*	18.4±0.2	7.5±0.4	2.5±0.2
1000	18.1±1.1	8.2±0.4	2.2±0.2
2000	25.3±2.6	16.0±1.3	1.5±0.0
3000	33.2±2.3	27.4±1.6	1.2±0.1
4000	33.4±3.8	36.3±1.7	0.9±0.2
5000	30.0±3.5	45.5±2.4	0.7±0.2

The data are the average of triplicate except one (*) with duplicate (mean ± S.D.).

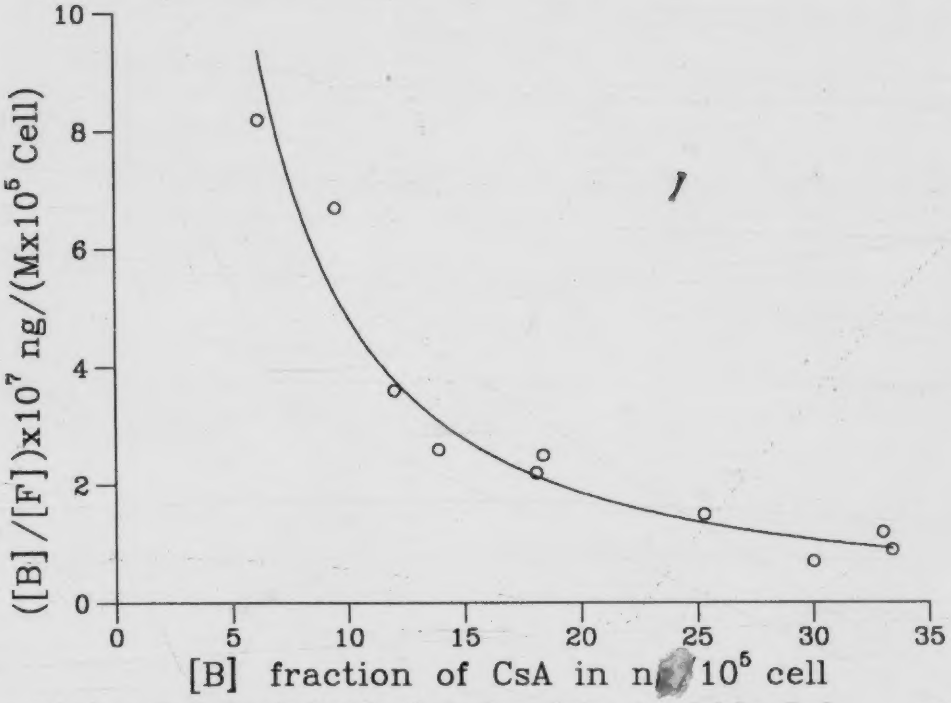


Figure 3.2 Scatchard plot for data in Table 3.2.

Table 3.3 High affinity of dissociation constants (K_d) for CsA-binding molecule complex in fibroblast cytosol.

Cell strain (passage no.)	Type of cell strain	K_d (x10^{-7} M)
HUGE-5 (P5)	Normal	2.3
HUGE-6 (P4)	Non-responder	3.2
HUGE-7a (P4)	Non-responder	2.4
HUGE-7b (P4)	Responder	2.3

Average of the above results is 2.6±0.4 (mean ± S.D.).

Table 3.4 Percentage of total cyclosporine A added to the culture system (6-well-plate) recovered in the medium after 1 h. incubation.

Medium	CsA distributions		
[CsA] ng/mL	Medium (no cell)	Medium (with cell)	In cells
5000	106%	106%	2.1%
4000	93.9%	93.9%	2.7%
3000	95.8%	95.8%	3.5%
2000	97.3%	97.3%	3.8%
1000	93.9%	93.9%	5.5%
800	117%	114.5%	7.1%
600	110.8%	106.4%	9.1%
400	115.2%	100.5%	9.4%
200	96.5%	88.2%	15.7%
100	94.7%	90.8%	18.8%

In columns (1) and (2), pipetting errors during experiments were considered to be responsible for those results > 100%.

3.3.4 Effect of ouabain on the uptake of cyclosporine A by fibroblasts.

No cytotoxicity was found after culturing the fibroblasts with ouabain (1×10^{-8} to 1×10^{-6} M) for 2 h. or 24 h. Although

Table 3.5 Effect of ouabain on cyclosporine A uptake by fibroblasts.

[Ouabain] in DMEM-10	Mean dpm ± S.D. 1st Cytosol Fraction	 2nd Cytosol Fraction
0	12857±3205	722±94
1×10^{-6}M	10327±3838	694±136
(Student's t-test)	($p=0.35$)	($p=0.75$)
0	13610±1791	779±118
5×10^{-7}M	14049±747	945±314
(Student's t-test)	($p=0.67$)	($p=0.36$)
0	10518±4220	663±239
1×10^{-7}M	11089±3715	703±267
(Student's t-test)	($p=0.85$)	($p=0.83$)
0	16014±4469	871± 67
1×10^{-8}M	15158±1782	896±190
(Student's t-test)	($p=0.73$)	($p=0.82$)

The cell strain was N606B, passage 5. The data were corrected with the cell numbers (225,000 cells/well) which are average of 10 wells with a S.D.= 4×10^4. NS (non-statistical difference) was accepted at $p \geq 0.05$.

ouabain precipitated some of the CsA in the medium (data not shown), this problem could be ignored since it did not affect the rate of uptake of CsA in the cells. Ouabain at the concentrations used did not interfere with the uptake of CsA by the fibroblasts (Table 3.5). Ouabain was found to have a half maximum inhibition of the Na^+-K^+-stimulated membrane ATPase in human erythrocytes at 1.3×10^{-7}M, but only 3.7×10^{-8}M is needed for half maximal inhibition of ion transport in the human (Csaky, 1973).

3.4 DISCUSSION

Our results showed that CsA was rapidly concentrated inside the cytoplasm of fibroblasts to an extent 30- to 40-fold greater than that in the extracellular medium (assuming the volume of the cytosol and the total cell volume is the same). From 79 to 85% of the total CsA taken up by the cells was located in the cytoplasm fraction. The high affinity CsA-binding molecule complex in the cytosol has a K_d 2.6×10^{-7} M. However, the K_d of the low affinity one(s) is almost infinite, which is considered as non-specific adsorption to cytosolic molecule(s). The uptake of CsA by the high affinity molecule is saturable at the concentration of CsA about 1000 ng/mL, while the low affinity one is non-saturable in up to 10 µg/mL CsA in the medium. The K_d for the high affinity CsA-binding site in fibroblasts is very close to the one measured in T lymphocytes (see above) and all of the present results are in agreement with those of Merker and Handschumacher (1984) and Fabre et al. (1986), who used lymphocytes from lymphoma or thymoma.

The Scatchard plot is a standard method for analysis of ligand binding studies. For a linear Scatchard plot, there is a single binding site for the ligand. The equation for that is (Scatchard, 1949; Rosenthal, 1967):

$$[B]/[F]=K(n[M]-[B]) \quad \ldots\ldots\ldots \quad (1)$$

where, [B] is the bound fraction of CsA in the cytosol;

[F] is the total free ligand concentrations;

K is the association constant ($K_d=1/K$);

n is the number of binding sites per macromolecule;

M is the total concentration of macromolecule available for binding.

If the Scatchard plot is a curve, it indicates there is more than one binding site for the ligand. This is either because there are multiple (not homogeneous) binding sites on a single acceptor molecule of interest or because the reaction system contains more than one kind of acceptor molecule. The latter is often the case in biological preparations in which whole cells or cell membranes are used (Zierler, 1989). This is also likely to be the case in our binding study system in which the whole cytosol was used.

A non-linear Scatchard plot may be resolved into two or more straight lines. Theoretically, the number of binding sites can be determined by trials to fit the curve to one, two, or three resolved line(s). Even had we assumed this method were applicable, it still shares an uncertainty common to similar empirical methods: if, within limits, the fit is acceptable to the investigator for m resolved lines, how does one know that there are not really m+1 resolved lines? However, it is impossible to resolve a curve into more than two straight lines by the graphical approach. The numerical curve regression method hardly gives acceptable result for a problem of more than three binding sites (Weder et al., 1974). If a non-linear Scatchard plot is obtained in our studies it is assumed to be due to two independent binding sites. Therefore, the Scatchard plot will be resolved into two straight lines which represent two binding groups. The equations of non-linear Scatchard plot for two binding groups are listed below (Rosenthal, 1967; Feldman, 1972):

$$[B]/[F]=K_1n_1[M_1]/(K_1[F]+1) + K_2n_2[M_2]/(K_2[F]+1) \quad \ldots(2.a)$$

$$\alpha X^2+\beta XY+Y^2+\gamma X+\delta Y=0 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2.b)$$

Where $X=[B]$;

$Y=[B]/[F]$;

$\alpha=K_1K_2$;

$$\beta = K_1 + K_2;$$

$$\gamma = -K_1K_2(n_1[M_1]+n_2[M_2]);$$

$$\delta = -K_1n_1[M_1]-K_2n_2[M_2].$$

The equation of 2.a or 2.b was resolved by computer programming for this study.

The ATPase inhibitor ouabain did not affect the uptake of CsA by the fibroblasts, suggesting that this is a passive process. This seems in conflict with the finding that the cells can concentrate it 30- to 40-fold over the extracellular solution. It indicates that the intracellular binding molecules (both high and low affinity ones) in the cells are very important in the accumulation of the drug in the cytoplasm. The action of CsA on T lymphocytes mainly involves blocking early activation events in the cells (Hess et al., 1988), whilst the effect of CsA on gingival fibroblasts is to interfere with connective tissue metabolism (Rateitschak-Pluss et al., 1983; Wysocki et al., 1983; Rostock, 1986). It is not clear whether CsA has the same initial site of action in all of these cells. If it does, what is it? Potential candidates are the ubiquitous cytosolic proteins, cyclophilin and/or calmodulin. Therefore, it would be interesting to know if the CsA-binding molecules in the fibroblast cytosol are the same as those found in lymphocytes. Furthermore, the physiological and biochemical functions of cyclophilin and calmodulin in collagen metabolism remain to be elucidated.

3.5 BIBLIOGRAPHY

COLOMBANI, P.M., ROBB, A., HESS, A.D. Cyclosporin A binding to calmodulin: a possible site of action on T lymphocytes. Science, 228:337-339, 1984.

CSAKY, T.Z. Drug affecting the exchange of material across cell membranes. In: Fundamental of cell pharmacology. S. Dikstein editor, Charles C. Thomas Publisher, Springfield, Illinois, U.S.A., 1973. pp. 342-344.

DALEY, T.D., WYSOCKY, G.P. Cyclosporine therapy. Its significance to the periodontist. J. Periodontol. 55:708-712, 1984.

DALGARNO, D.C.; HARDING, M.W., LAZARIDES, A.; HANDSCHUMACHER, R.E.; ARMITAGE, I.M. ^{1}H NMR studies on bovine cyclophylin: Preliminary structural characterization of this specific cyclosporine A binding protein. Biochem. 25:6778-6784, 1986.

FABRE, I., FABRE, G., LENA, N., CANO, J.-P. Kinetics of uptake and intracellular binding of cyclosporine A in RAJI cells, in vitro. Biochemical Pharmacology. 35:4261-4266, 1986.

FELDMAN, H.A. Mathematical theory of complex ligand-binding system at equilibrium: Some methods for parameter fitting. Analyt. Biochem. 48:317-338, 1972.

HANDSCHUMACHER, R.E., HARDING, M.W., RICE, J. DRUGGE, R.J. Cyclophylin: a specific cytosolic binding protein for cyclosporin A. Science, 226:544-547, 1984.

HESS, A.D., ESA, A.H. COLOMBANI, P.M. Mechanism of action of cyclosporine: Effect on cells of the immune system and on subcellular events in T cell activation. Transplantation Proceedings. 20, Suppl. 2:29-40, 1988.

LEGRUE, S.J. ET AL., FRIEDMAN, A.W., KAHAN, B.D. J. Binding of cyclosporine by human lymphocytes and phospholipid vesicles. J. Immunol. 131:712-718, 1983.

LEGRUE, S.J.; TURNER, R.; WEISBRODT; DEDMAN, J.R. Does the binding of cyclosporine to calmodulin result in immunosuppression? Science. 23:68-71, 1986.

MERKER, M.M., HANDSCHUMACHER, R.E. Uptake and nature of the intracellular binding of cyclosporin A in a murine thymoma cell line BW5147. J. Immunol. 132:3064-3070, 1984.

RATEITSCHAK-PLÜSS, E.M., HEFTT, A., LORTSCHER, R., THIEL, G. Initial observation that cyclosporin-A induces gingival enlargement in man. J. Clin. Periodontol. 10:237-246, 1983.

ROSTOCK, M.H., FRY, H.R., TURNER, J.E. Severe gingival overgrowth associated with cyclosporine therapy. J. Periodontol. 57:294-299, 1986.

ROSENTHAL, H.E. A graphic method for the determination and presentation of binding parameters in a complex system. Analyt. Biochem. 20:525-532, 1967.

SCATCHARD, G. The attractions of proteins for small molecules and ions. Ann. New York Acad. Sci. 51:660-672, 1949

WEDER, H.G., SCHILDKNECHT, J., LUTZ, R.A., KESSELRING, P. Determination of binding parameters from Scatchard plots. Theoretical and practical considerations. Eur. J. Biochem. 42:475-481, 1974.

WYSOCKI, G.P., GRETZINGER, H.A., LAUPACIS, A. ULAN, R.A. STILLER, C.R. Fibrous hyperplasia of gingiva: a side effect of cyclosporin-A therapy. Oral Surg. Oral Med. Oral Path. 55:274-278, 1983.

ZIERLER, K. Misuse of nonlinear Scatchard plots. TIBS. 14:314-317, 1989.

CHAPTER IV

CYCLOSPORINE A DEPRESSES THE DESTRUCTION OF FIBRILLAR COLLAGEN BY GINGIVAL FIBROBLASTS *IN VITRO*

4.1 INTRODUCTION

Immunosuppressive treatment with cyclosporine A (CsA) can cause gingival hyperplasia and other side-effects which are described elsewhere (Wysocki et al., 1983; Bennett and Norman, 1986). Although an increase in the amount of collagen is one of the major features of CsA-associated gingival overgrowth (Wysocki et al., 1983), little is known about the effects of CsA on collagen catabolism.

Collagens are a group of major structural proteins in all connective tissues. Their degradation can proceed by two main pathways, i.e., extracellular and intracellular (Woolley, 1984). The former involves several matrix metalloproteinases (MMPs) that degrade collagen fibrils into different sizes of fragments at neutral pH. The latter requires that fibroblasts phagocytose fibrillar collagen fragments which have been truncated by some preliminary extracelluar degradations. These fragments are then digested in the acid environment within phagolysosomes (for recent reviews, see Sodek and Overall, 1988; Birkedal-Hansen, 1988).

Collagenase (matrix metalloproteinase I, MMP-I) is an enzyme with a high specificity for cleaving the triple helical collagen molecule at a Gly-Ile peptide bond (residues 775: glycine and 776: isoleucine) in the α_1(I) chain and at Gly-Leu (leucine) in the α_2(I) chain at neutral pH, resulting in 3/4- and 1/4-length fragments of type I collagen, TC_A (at the N-terminal end) and TC_B (Figure 4.1) (Woolley, 1984). Since the monomeric α chains of type I collagen can crosslink into

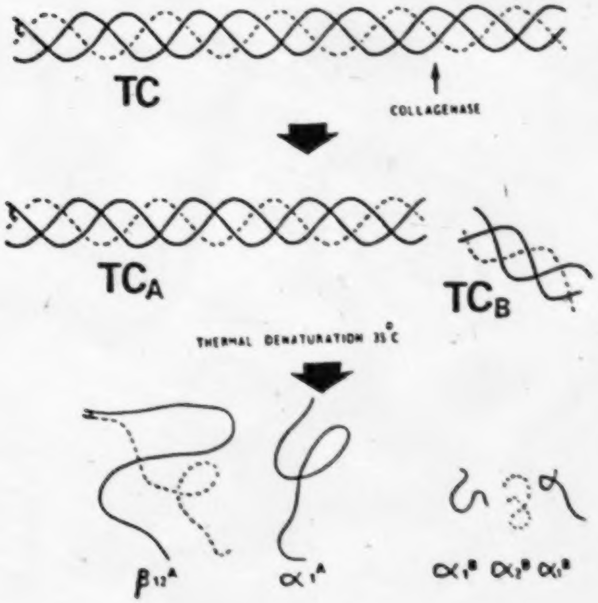

TC
COLLAGENASE
TC_A
TC_B
THERMAL DENATURATION 35°C
β_{12}^A
α_1^A
α_1^B α_2^B α_1^B

Figure 4.1 Diagrammatic illustration showing cleavage of native type I collagen molecule (TC) by collagenase at 25°C. Two helical fragments, TC_A and TC_B, three quarters and one quarter the length of the original molecule, respectively, are produced.

dimers and trimers via intramolecular crosslinks at the N-terminal end, which are referred to as ß- ($ß_{11}$ and $ß_{12}$) and γ-components, respectively. The denatured TC 3/4 fragments are also named as α_1^A and α_2^A (or α_1 3/4 and α_2 3/4) for monomers; $ß_{11}^A$ and $ß_{12}^A$ (or $ß_{11}$ 3/4 and $ß_{12}$ 3/4) for dimers and γ^A for the trimer (Miller, 1984; Woolley, 1984). Collagenase is usually produced as a zymogen (procollagenase) by mammalian cells such as fibroblasts (Stricklin et al., 1977 and 1978). The zymogen is usually activated in vitro by trypsin (Harper et al., 1971) or organomercurial compounds (Sellers et al., 1977), but the precise mechanism of activation of latent collagenase in vivo is unknown (for a review, see Harris et al., 1984).

Many types of cell can produce procollagenase. Among them, fibroblasts are the main source in the soft connective tissue (Woolley, 1984). Gingival fibroblasts usually produce little or no MMPs without stimulation (P. G. Scott, unpublished observation). The agents which can be used for stimulating fibroblasts to produce procollagenase in vitro include phorbol myristate acetate (PMA) [(also known as 12-O-tetradecanoyl-phorbol-13-acetate (TPA)] (Brinckerhoff et al., 1979; Aggeller et al., 1984a), concanavalin A (ConA) (Nakano and Scott, 1985), mononuclear cell factor (Dayer et al., 1980) or human recombinant interleukin-1α (hrIL-1α) or hrIL-1ß (Ito et al., 1988; Richards and Rutherford 1988; Katsura et al., 1989), and human recombinant tumour necrosis factor (hrTNF)-(α)/(ß) (Duncan and Berman, 1989). Collagen fibrils (both denatured and native) were also reported to increases production of the enzyme (Biswas and Dayer, 1979). Only the last three factors (IL-1, collagen fibrils and TNF) are physiologically relevant stimulators.

Interleukin-1 (IL-1) is a group of polypeptides which mediate a wide range of biological activities such as inflammation, immune regulation, and the function of fibroblasts (for recent reviews, see Oppenheim et al., 1986; Dinarello, 1988). They are mainly produced by activated macrophages in response to infection, injury or antigenic challenge (Dinarello, 1984). Other sources of IL-1 are from epidermal, epithelial, lymphoid and vascular tissues (Oppenheim et al., 1986; Dinarello, 1988). There are two types of IL-1, namely IL-1α and IL-1ß; and the precursors (31 kDa) of IL-1α and IL-1ß are coded by two separate genes, both located on chromosome 2 (Dinarello, 1988). The mature peptide (IL-1α: pI 5.0, M.W. 17 kDa; IL-1ß: pI 7.0, M.W. 17 kDa) and other smaller peptides are produced from the precursor by an intracellular serine proteinase (Auron et al., 1984; Lomedico, et al., 1984). The precursors and 22 kDa forms of IL-1, most of which are IL-1α, are found to be associated with cells, either intracellularly (for 31 kDa only) or membrane-bound (for both 31 kDa and 22 kDa) (Lepe-Zuniga and Gery, 1984; Matsushima et al., 1986). Human IL-1α and IL-1ß only have 26% homology in their amino acid sequence and 45% in their nucleotide sequences (March et al., 1985). Although IL-1α and

IL-1ß are biochemically distinct, they show an almost identical spectrum of biological activity, both in vivo and in vitro (Dinarello, 1988). This may be explained by the fact that they share the same receptor (with a M.W. about 80 kDa) on the cell membrane (Dower et al., 1986; Dower and Urdal, 1987).

IL-1 plays an important role in the regulation of connective tissue metabolism in tissue trauma and inflammation (for reviews, see Dinarello, 1984; Oppenheim, 1986; Dinarello, 1988). Each type of IL-1 (IL-1α and IL-1ß) can increase the proliferation of fibroblasts, synthesis of collagen, collagenase and prostaglandin-E in the cells (Richards and Rutherford, 1988; Ito et al., 1988; Duncan and Berman, 1989). IL-1α can suppress the biosynthesis of tissue inhibitor of metalloproteinases (TIMP) (Ito et al., 1988). IL-1α or IL-1ß increase the connective tissue degradation function of fibroblasts more than 10-fold greater than they enhance the synthesis function of the cells (Duncan and Berman, 1989).

In the present studies on the mechanism of CsA-induced gingival hyperplasia (CsA-GH), the effect of CsA on the degradation of fibrillar collagen by gingival fibroblasts was evaluated. The stimulators used for collagenase production by fibroblasts were type I bovine tendon insoluble collagen fibrils, ConA, hrIL-1α and hrIL-1ß added into the culture system. We report here the first direct evidence that CsA at therapeutic concentrations can interfere with the capacity of fibroblasts to degrade fibrillar collagen, thus suggesting a mechanism for the hyperplasia.

4.2 MATERIALS AND METHODS

4.2.1 Culture of gingival fibroblasts.

The materials and methods for cell culture are described in detail in chapter II. Human gingival fibroblast cell strains used in the present studies were CsA-GH responder cell strains (CSA-8, CSA-11 and CSA-12) and normal cell strains (N606B, N1106, N1806, HUGE-12 and HUGE-15). The preparations of the growth medium, i.e., Dulbecco's modified Eagle medium containing 10% (v/v) fetal calf serum (DMEM-10), and CsA containing medium were also described in chapter II. All cell cultures were carried out in a Forma or National CO_2 incubator which was always set at 5% CO_2, 95% air, 1 atmosphere, 37°C, and 95% humidity.

4.2.2 Fluorescent labelling of collagen fibrils.

Insoluble bovine Achilles tendon type I collagen (Sigma) was labelled with fluorescein isothiocyanate (FITC) (Sigma) (Steven and Lowther, 1975). After washing 4 times each with water, methanol (50% v/v) and acetone to remove as much unbound fluor as possible, the collagen was resuspended at about 1 mg/mL in 50% methanol containing 1% (v/v) glacial acetic acid by repeating homogenization for a few seconds using a Polytron homogenizer. Any large pieces of collagen were removed by centrifugation at 1000 rpm for 2 min. An aliquot (100 µL) of this suspension was added to each well of the 24-well tissue culture plates which were then allowed to dry uncovered in a sterile atmosphere for 1 hour.

4.2.3 Culture of fibroblasts on fluorescent collagen fibrils.

In preparation for addition of cells, 1 mL of phosphate-buffered saline (PBS) was added to each well and the plates incubated at 37°C for several days with daily changes of buffer, until the rate of release of fluorescence had declined to about 10 units/day (arbitrary units). Each well was seeded with $2x10^5$ cells in 1 mL of DMEM-10 and put into the incubator overnight. Fresh medium containing CsA (200 to 800 ng/mL) was added and replaced each day. Released fluorescence (excitation wavelength 490 nm, and emission 520 nm) was measured in the culture medium samples after brief centrifugation. The instrument (Perkin-Elmer LS-5 luminescence spectrophotometer) was zeroed using medium recovered from wells incubated without added cells. After 4 days, cells were released from the plates by incubation with 0.5 mL of a solution of bacterial collagenase (25 units: Worthington CLSPA) in Tris buffer (0.2 M-KCl/0.005 M-$CaCl_2$/0.02%, w/v, sodium azide/0.05 M Tris-HCl, pH 7.4) for 2 hour at 37°C, followed by 0.5 mL trypsin (0.25%, w/v; Worthington, 1:250) for 4 min at room temperature (R.T.), and counted on a haemocytometer. The residual FITC-collagen (1 mL) in the supernantant of the collagenase and trypsin digestion was measured as above.

4.2.4 Enzyme digestion of the fluorescent collagen fibrils.

Alternatively, for use as a suspension, FITC-collagen, after washing 4 times each with PBS, 50% methanol and acetone, was suspended in DMEM-0 (serum free medium) instead of 50% methanol. Glacial acetic acid (1% v/v) was added and samples were homogenized as above. Final FITC-collagen suspension, at about 3-fold dilution of the one used for coating on the cell culture plates, was in DMEM-0 supplemented with 0.02% (w/v) sodium azide. This suspension was aliquoted into microfuge tubes (0.5 mL/tube) and stored at 4°C in the dark for use (maximum 1 week).

Before adding enzyme containing samples and CsA into the FITC-collagen containing tubes, the background of the fluor released into the supernatants in all tubes were measured. The tubes were centrifuged (Microfuge, Beckman) and the supernatants were discarded. In order to measure the direct effect of CsA on activity of the activated collagenase, the FITC-collagens in the tubes were resuspended in 0.5 mL of DMEM-0 and 10 µL of the concentrated (by ammonium sulfate precipitation, see below) and activated (by TPCK trypsin, see below) collagenase containing sample from non-CsA treated normal gingival fibroblast culture medium was directly added into FITC-collagen suspensions and incubated with CsA (200 to 800 ng/mL) at 37°C with 360° slow rotation in the dark for 2 hours. The samples were briefly centrifuged and the fluorescence in the supernatants measured as above.

4.2.5 Preparation of collagenase-containing medium and assays for enzyme activities.

The stock ConA (Pharmacia) solution was made at 2.0 mg/mL in PBS and sterilized by passing through a Millex-GS™ filter (0.22 µm) (Millipore). The sterile hrIL-1α and hrIL-1ß (500 half-maximum units per 50 µL) were from Collaborative Research Inc., MA., USA. The half maximum unit of hrIL-1α or hrIL-1ß is calculated as the reciprocal of the dilution which causes 50% stimulation in the lymphocyte activation factor (LAF) Assay (Collaborative Research Inc. product description). The stock solution of lactalbumin hydrolysate (DIFCO) was prepared at 25% (w/v) in PBS and sterilized by autoclave. The medium used for collagenase preparation was made from DMEM-0 supplemented with 25 µg/mL ConA, 0.5% (w/v) lactalbumin hydrolysate, with or without 800 ng/mL CsA; or using 1:30,000 dilution (i.e., 0.33 half maximum unit per mL, final concentration) of the commercially available hrIL-1α or hrIL-1ß instead of ConA. The stock solutions of trypsin (Worthington, TPCK) and soybean trypsin inhibitor (Sigma) were freshly prepared at 1 mg/mL and 10 mg/mL in Tris buffer, respectively.

Type I acid soluble collagen (ASC) purified from bovine skin (Scott and Goldberg, 1983) was dissolved in 0.1 M acetic acid at 2 mg/mL and gently stirred at 4°C overnight. The dissolved ASC was dialysed against 1 litre of 0.01 M acetic acid at 4°C for three days with daily changes of 1 L of fresh acetic acid. The final concentration of the stock ASC solution was measured with Biuret assay (Itzhaki and Gill, 1964) with a spectrophotometer (Gilford) at $A_{300\ nm}$. This was stored at -20°C until used.

To assess the effects of CsA on gingival fibroblasts producing collagenase, the cells were grown to confluence in 75 cm^2 tissue culture flasks, washed with PBS and then fed with the fresh serum free medium (12 mL/flask). Enzymes in the medium, which was collected each day for three days, were concentrated by precipitation with ammonium sulfate (Ultra Pure^{TM}, from Schwarz/Mann Biotech) at 60% saturation and redissolved in a small volume of Tris buffer (Scott et al., 1983). The samples were activated by proteolysis with the stock trypsin solution at 1:100 dilution (v/v, final concentration) for 15 min at R.T., followed by addition of the soybean trypsin inhibitor stock solution at 1:100 for 15 min in ice bath to terminate the trypsin activity (Scott et al., 1983). The enzyme activities (general proteinases and collagenase) in these samples were assayed in duplicate (see below). If the enzyme activity in the sample was too high, appropriate dilution with Tris buffer was made. These concentrated and activated samples were used for collagenase and proteinase activity assay (see below).

For the collagenase activity assay, the method was derived from Berman et al. (1973) and Scott et al. (1983). The collagenase substrate, i.e., the working ASC solution (1 mg/mL, pH 7.4), was prepared from the stock solution by adding an equal volume of 2-fold concentrated Tris buffer and degassing for 15 min at R.T. Glass capillaries (length = 75 mm, I.D. = 1.1 - 1.2 mm, wall thickness = 0.20 mm ± 0.02 mm, non-heparinized; Fisher) were partially filled with this solution and sealed at one end with Critoseal and temporarily covered at the other ends with Parafilm. The capillaries were transferred to the 37°C warm room for 1 to 2 days to allow the collagen to gel and became opaque. The top of the collagen in each capillary was marked. Samples (with or without dilution depending on the enzyme activities in the samples) were gently transferred to the capillaries (5 µL/capillary) with a Hamilton microsyringe and the tops were sealed with wax. Incubation was carried out in the warm room for 16 hours. The amount of digestion was calculated as microgram/16 h.

In a second method, 5% SDS-PAGE (sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis) (Mechanic, 1979), was used to measure the collagenase activities in the samples from cell cultures in the conditioned medium containing hrIL-1α or hrIL-1ß. Briefly, 5% SDS gels were prepared and stood at R.T. overnight. The collagenase substrate in microfuge tubes (50 µL/tube) was incubated with 50 µL of the samples (with or without further dilution) at R.T. for 30 min to 16 h. (depending on the enzyme activities in the samples). The digestion was terminated by adding 100 µL of SDS-sample buffer containing 10% 2-mercaptoethanol and 0.001% (w/v) bromphenol blue followed by boiling for 5 min. The molecular weight standard (low M.W., Pharmacia) containing lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase B with M.W. 14,400, 21,500, 31,000, 45,000, 66,000 and 92,500 dalton, respectively, was prepared by pipetting 10 µL of the standard into 95 µL of the SDS-sample buffer. The "no enzyme control" was prepared with Tris buffer instead of sample in the same way as above. After brief centrifugation, 50 µL each of the above samples and standards was pipetted onto each lane of the gel which was run at 50 V for 30 min, followed by 500 V for 3 h. The gel was then stained with Coomassie Blue (0.125%, w/v, Coomassie Blue R250 in 7.5% acetic acid and 50% methanol) for 1 hour at R.T. followed by destaining with 5% methanol in 7.5% acetic acid for 2 to 3 days. The bands resolved in the gel were quantified by scanning with a Joyce-Loebel scanner at 530 nm.

The general proteinase activity assay was modified from Chavira et al. (1984). Azocoll (Sigma) was weighed into microfuge tubes (3 mg/tube). The concentrated enzyme-containing samples, either non-activated, activated (see above) or boiled and activated samples (0.3 mL/tube) and Tris buffer (1.2 mL/tube) were added. The background tubes were Azocoll (3 mg) with 1.5 mL buffer only. After incubation at 37°C with 360° slow rotation for 16 hours, one drop of glacial acetic acid was added to each tube which was then centrifuged for a few seconds (Beckman Microfuge). The red colour in the supernatants was read at 520 nm (Gilford).

Fibroblasts were not trypsinized off the culture flasks after ConA-treatment. After exposing the cellls to the serum-free medium for 3 days, they were cultured in DMEM-10 for 5 to 7 days. The cells could then be harvested by trypsinization. The number of cells were counted as before (Chapter II).

4.3 RESULTS

4.3.1 Effect of cyclosporine A on digestion of fibrillar collagen.

In all seven cell strains tested, CsA caused measurable depression of the capacity of the cells to degrade fibrillar collagen (Table 4.1). Results shown in Table 4.1 are averages from 4 wells, except for N606B (6 wells). This cell strain (N606B) gave very weak digestion and only showed a significant effect of CsA at 800 ng/mL. This may be due to the cells being too old (passage 9). The effects were seen (varying from 11% to 64% inhibition) at 200 ng/mL of CsA in four other cell strains. The attachment of cells of two strains (CSA-12 and N1806) appeared to be adversely affected by CsA at 800 ng/mL of CsA, as reflected in the reduced number of cells recovered after four days. The other cell strains appeared to tolerate such conditions well. At the end of the experiments, 80 to 95% of the collagen remained in the wells, as determined by measuring the fluorescence released by sequential digestion with collagenase and trypsin (data not shown).

Table 4.1 Effects of cyclosporine A on digestion of fibrillar collagen.

Cell strain (passage)	[CsA] ng/mL	Accumulated Fluorescence	Cellsx10^5 per well	Fluorescence per 10^5 cells
CSA-8	0	148.4±13.4	2.1±0.3	71.0± 6.4
(4)	200	123.7± 1.6	2.1±0.1	59.2± 0.8 (p=0.010)
	400	99.6±10.5	2.1±0.3	47.9± 5.1 (p=0.001)
	800	96.7± 9.2	1.9±0.2	52.3± 5.0 (p=0.004)
CSA-12	0	106.6±2.8	0.45±0.1	237.0± 6.3
(3)	200	55.7± 7.1	0.66±0.2	84.4±10.8 (p<0.001)
	400	33.6± 5.2	0.46±0.2	73.0±11.2 (p<0.001)
	800	13.0± 1.4	0.26±0.1	49.8± 5.2 (p<0.001)
N1106	0	171.5± 7.2	0.65±0.1	263.8±11.1
(7)	200	144.9±13.9	0.66±0.1	219.5±21.1 (p=0.010)
	400	131.8± 8.8	0.80±0.1	164.8±11.1 (p<0.001)
	800	133.6± 1.9	0.75±0.1	178.1± 2.6 (p<0.001)

(Table 4.1 contd.)

Cell strain (passage)	[CsA] ng/mL	Accumulated Fluorescence	Cellsx10^5 per well	Fluorescence per 10^5 cells
N606B	0	14.7± 2.6	0.57±0.2	25.8± 4.5
(9)	200	12.7± 1.4	0.57±0.2	22.5± 2.6 (p=0.140*)
	400	14.3± 3.5	0.54±0.2	26.8± 6.6 (p=0.780*)
	800	10.8± 1.2	0.55±0.2	19.6± 2.1 (p=0.012)
HUGE-12	0	569.3±34.2	0.97±0.3	586.9±35.3
(4)	800	373.9±15.3	1.05±0.2	356.1±14.6 (p<0.001)
HUGE-15	0	399.3±52.6	1.32±0.2	302.5±39.9
(3)	800	266.7±35.2	1.21±0.2	220.4±29.1 (p=0.016)
N1806	0	59.2± 2.6	1.26±0.3	47.0± 2.0
(5)	200	60.5± 2.8	1.45±0.2	41.7± 1.9 (p=0.010)
	400	26.6± 0.4	0.98±0.3	27.1± 0.4 (p<0.001)
	800	17.3± 3.1	0.46±0.1	37.6± 6.8 (p=0.037)

There was no significant difference between results at [CsA] given and 0 ng/mL for N606B (Student's t-test, p≥0.05*). In all other cases the depression of collagen digestion by CsA was significant.

4.3.2 Direct effect of cyclosporine A on collagenase activity.

Cyclosporine A was not found to be an inhibitor of gingival fibroblast collagenase per se but rather to enhance the activity of the activated enzyme at 400 and 800 ng/mL of CsA (Table 4.2). However, CsA did not activate the procollagenase to an active form, since there was no enzyme activity in the concentrated and non-activated samples from the medium of "CsA + DMEM-0" treated cells (CSA-8 in the interleukin-1 study), which was seen to have "auto-release" of collagenase after activating with trypsin (see below, but for non-activated samples, data not shown).

4.3.3 Effect of cyclosporine A on concanavalin A stimulation of fibroblast production of proteinases and collagenase.

Fibroblasts rounded up after 10 to 24 hours' treatment with ConA (Figure 4.2), which was a sign of the activity of collagenase production (Aggeller et al., 1984b). Non-producing cells did not show morphological changes (unpublished observation). In the hrIL-1α- or hrIL-1ß-treated fibroblasts (see below), this phenomenon was also seen (data not shown). CsA did not inhibit the morphology changes induced by ConA or hrIL-1α or hrIL-1ß (data not shown). There was no significant difference in the number of cells among the groups, as determined by counting the number of cells after reculturing

Table 4.2 Effect of CsA on capacity of the activated collagenase-containing samples (10 µL) to digest fibrillar collagen.

[CsA] (ng/mL)	Fluorescence
0	26.1± 11.5
200	102.6±147.5 (p=0.420*)
400	368.2± 97.6 (p=0.004)
800	462.3± 64.1 ($p<0.001$)

The data are the average of triplicate assays which were subtracted with the control ("FITC-collagen + CsA 800 ng/mL" without enzyme) and expressed as mean ± S.D. Student's t-test was performed for CsA at given concentrations and 0 ng/mL. Statistical difference is accepted at $p \le 0.05$. NS* means non-statistical difference.

in DMEM-10 for 5 to 7 days. Thus, the cells rounded up after ConA treatment were still alive.

No general proteinase or collagenase activity could be detected in most of the samples of medium not incorporating ConA in the five cell strains tested, except at day 3 of HUGE-15 which showed slight enzyme activity without stimulation (Tables 4.3.a and 4.3.b). ConA stimulated the release of general proteinase and collagenase activity in all five cell strains tested, although in three no effect could be detected until day 2 (Tables 4.3.a and 4.3.b). In most cases, at each day when proteinase and collagenase were detectable there was a marked suppression by CsA in most cell strains tested. The general proteinase activities were not completely parallel to the collagenase activity in the same samples. In three cell strains (CSA-11 at day 2, CSA-12 at day 3, HUGE-12 at day 2 and 3) CsA did not inhibit of general proteinase activity. Trypsin (TPCK, 1 mg/mL) did not give measurable digestion either in the assays of 5% SDS-PAGE, capillary or fluorescence-labelled fibrillar collagen (data not shown).

(a) Cells in DMEM-0 (day 2)

(b) Cells in DMEM-0 + ConA (25 µg/mL) (day 2).

Figure 4.2 Fibroblast (N1106, passage 5) morphology changes in response to concanavalin A stimulation (Magnification x100, phase contrast).

Table 4.3.a Effect of cyclosporine A (800 ng/mL) on general proteinase activity (Azocoll assay).

Cell strain (passage)	Medium (DMEM-0)	Azocoll digestion (A_{520nm})		
		Day 1	Day 2	Day 3
CSA-8	+No drug	0.004	0.006	0.006
(5)	+CsA	0.002	0.003	0.003
	+ConA	0.001	0.651	0.581
	+ConA+CsA	0.000	0.099	0.053
CSA-11	+No drug	0.025	0.036	0.003
(5)	+CsA	0.039	0.065	0.000
	+ConA	0.517	0.831	0.875
	+ConA+CsA	0.301	0.840	0.507
CSA-12	+No drug	0.037	0.000	0.000
(4)	+ConA	0.002±0.004	0.001±0.003	0.000±0.001
	+ConA+CsA	0.001±0.001	0.000±0.000	0.005±0.006

(Table 4.3.a contd.)

Cell strain (passage)	Medium (DMEM-0)	Azocoll digestion (A_{520nm}) Day 1	Day 2	Day 3
HUGE-12	+CsA	0.042	0.000	0.061
(5)	+ConA	0.351±0.253	0.489±0.055	0.637±0.060
	+ConA+CsA	0.015±0.002	0.437±0.084	0.502±0.176
HUGE-15	+No drug	0.000	0.000	0.107
(4)	+CsA	0.000	0.000	0.027
	+ConA	0.000±0.000	0.383±0.124	0.726±0.033
	+ConA+CsA	0.001±0.000	0.001±0.001	0.027±0.021

In CSA-12 and HUGE-12, there was no "no drug" control. The samples in the "+ConA" and the "+ConA+CsA" groups for HUGE-12 and HUGE-15 were from duplicate flasks; while for CSA-12 they were from 4 and 2 replicate flasks, respectively. The rest of the samples had single flasks for each group. Duplicate assays were done for each flask's sample. All the data were substracted with "boiled control" (see Material and Methods) and the results are presented as Mean ± S.D. All the digestions were over 16 hours at 37°C. No Student's t-test was done for this table.

Table 4.3.b Effect of cyclosporine A (800 ng/mL) on collagenase activity in fibroblast medium (Capillary assay).

Cell strain	Medium	Collagen digested (µg/16h.)		
(passage)	(DMEM-0)	Day 1	Day 2	Day 3
CSA-8	No drug	0.0±0.0	0.0±0.0	0.0±0.0
(5)	+CsA	0.0±0.0	0.0±0.0	0.0±0.0
	+ConA	0.0±0.0	11.9±0.5	10.4±0.0
	+ConA+CsA	0.0±0.0	8.3±0.0	6.8±0.5
			(p=0.095*)	(p=0.022)
CSA-11	No agent	0.0±0.0	0.0±0.0	0.0±0.0
(5)	+CsA	0.0±0.0	0.0±0.0	0.0±0.0
	+ConA	5.6±0.4	8.0±0.4	7.2±0.0
	+ConA+CsA	2.4±0.0	4.0±0.4	3.2±0.4
		(p=0.005)	(p=0.011)	(p=0.004)
CSA-12	No drug	0.0±0.0	0.0±0.0	0.0±0.0
(4)	+ConA	0.0±0.0	4.0±1.0	5.6±0.6
	+ConA+CsA	0.0±0.0	0.0±0.0	0.5±1.1
			(p<0.001)	(p<0.001)

(Table 4.3.b contd.)

Cell strain (passage)	Medium (DMEM-0)	Collagen digestion (μg/16h.) Day 1	Day 2	Day 3
HUGE-12	+CsA	0.0±0.0	0.0±0.0	0.0±0.0
(5)	+ConA	0.0±0.0	4.0±1.3	7.2±0.6
	+ConA+CsA	0.0±0.0	4.0±0.4 (NS)	2.4±0.0
			($p=0.680^{*}$)	(p=0.002)
HUGE-15	No drug	0.0±0.0	0.0±0.0	2.4±0.0
(4)	+CsA	0.0±0.0	0.0±0.0	2.4±0.0
	+ConA	0.0±0.0	7.2±0.8	9.6±0.4
	+ConA+CsA	0.0±0.0	0.0±0.0	7.2±0.0
			($p<0.001$)	(p=0.017)

The samples are the same as those in Table 4.2. The data (Mean ± S.D.) are average of duplicate assays for each sample in every group, except for HUGE-12 with triplicate for day 2 of the "+ConA+CsA", day 3 of the "+ConA" and the "+ConA+CsA" groups; with 4 replicate assays for day 2 of the "+ConA" group. Student's t-test was carried out for "DMEM-0+No drug" with "DMEM-0+ConA" and "DMEM-0+CsA" with "DMEM-0+ConA+CsA". Non-statistical difference (NS) is accepted at $p \geq 0.05$.

4.3.4 Effect of cyclosporine A on human recombinant interleukin-1α or interleukin-1ß stimulation of collagenase production by fibroblasts.

After culture of gingival fibroblasts in DMEM-10 to confluence, exposure of the cells to hrIL-1α or IL-1ß at 0.33 half maximum unit/mL for 3 days had a slight effect on the proliferation of fibroblasts (Table 4.4). Both an increase (in CSA-12) and a decrease (in CSA-8) the number of cells were seen. In CSA-12 this effect of hrIL-1α or IL-1ß was not seen in the presence of CsA (800 ng/mL)

In the CSA-8 samples from day 2 and 3, the original concentrated and activated medium had very strong collagenase activity which resulted in digestion out of the linear range in the collagenase assay by either the capillary method or using 5% SDS-PAGE (data not shown). Therefore, further dilution of the samples was required before repeating the SDS-PAGE for collagenase assays.

Human recombinant IL-1α or hrIL-1ß stimulated fibroblasts (CSA-12 and CSA-8, both in passage 5) to produce collagenase (Table 4.5, Figures 4.3 and 4.5). In CSA-12, the "capillary assay" for collagen digestion was done by using samples without dilution for 16 h at 37°C. In this experiment, CSA-12 had no measurable collagenase activity without hrIL-1α or hrIL-1ß and the stimulation effect was not apparent until day 2 (Figures 4.3 and 4.4.b). In CSA-8, the assay for collagen digestion was done by incubating samples for 16 h at 37°C and the samples had no dilution for day 1, 2-fold dilution for day 2 and 10-fold dilution for day 3, respectively. CSA-8 had detectable collagenase activity without any stimulation and the hrIL-1α or hrIL-1ß effects were seen in all three days' culture (Table 4.5, Figures 4.5 and 4.6.a).

CsA enhanced the activated collagenase activity (Table 4.4, Figures 4.5 and 4.4.a), which was in agreement with the previous finding, but not the non-activated one (data not shown). CsA inhibited hrIL-1α- but not hrIL-1ß-stimulated production of collagenase (Figures 4.3, 4.5, 4.6.b and 4.6.c). The results of scanning the gel are presented in Figures 4.6.a, 4.6.b and 4.6.c.

Table 4.4 Number of cells (x10^6/flask) after 3 days' treatment with human recombinant interleukin-1α or interleukin-1ß.

(Group no.) DMEM-0	Cell strains CSA-12 (passage 4)	 CSA-8 (passage 5)
(1) No agent	2.3	5.0
(2)+CsA	2.3	5.0
(3)+hrIL-1α	2.4 ± 0.1 ($p=0.050^*$)	4.0 ± 0.3 ($p<0.001$)
(4)+hrIL-1α+CsA	2.3 ± 0.2 ($p<0.001$)	4.4 ± 0.1 ($p<0.001$)
(5)+hrIL-1ß	2.8 ± 0.1 ($p<0.001$)	4.6 ± 0.1 ($p<0.001$)
(6)+hrIL-1ß+CsA	2.2 ± 0.1 ($p=0.780^*$)	4.3 ± 0.1 ($p<0.001$)

The average number of cells (mean ± S.D.) was from two flasks except group (1) and (2) which had single flask, respectively. Group (3) and (5) were compared with group (1); group (4) and (6) with (2) by Student's t-test. *Non-statistical difference at $p \geq 0.05$.

Table 4.5 Effect of cyclosporine A (800 ng/mL) on collagenase activity in fibroblast medium (Capillary assay).

Cell strain (passage)	Medium (DMEM-0)	Collagen digested (μg/16h/1x10^6 cells) Day 1	Day 2	Day 3
CSA-12	(1) No drug	0.0±0.0	0.0±0.0	0.0±0.0
(5)	(2)+CsA	0.0±0.0	0.0±0.0	0.0±0.0
	(3)+hrIL-1α	0.0±0.0	7.2±1.2	8.4±0.9
	(4)+hrIL-1α+CsA	0.0±0.0	0.0±0.0 ($p<0.001$)	1.9±2.0 ($p<0.001$)
	(5)+hrIL-1ß	0.0±0.0	0.0±0.0	1.9±2.0
	(6)+hrIL-1ß+CsA	0.0±0.0	7.0±2.1	10.2±1.9

(Continue Table 4.5)

Cell strain (passage)	Medium (DMEM-0)	Collagen digested ($\mu g/16h/1x10^6$ cells) Day 1	Day 2	Day 3
CSA-8	(1) No drug	0.0±0.0	5.2±1.4	0.0±0.0
(5)	(2)+CsA	0.0±0.0	3.1±0.0	0.0±0.0
	(3)+hrIL-1α	6.3±1.8	6.4±0.7	3.6±0.6
	(4)+hrIL-1α+CsA	0.0±0.0	4.0±1.0	0.0±0.0
		($p<0.001$)	($p=0.003$)	($p<0.001$)
	(5)+hrIL-1ß	3.6±0.9	6.4±0.5	2.8±0.6
	(6)+hrIL-1ß+CsA	3.5±1.2	7.3±0.0	2.7±0.5
		($p=0.900^*$)	($p=0.064^*$)	($p=0.65^*$)

The data (Mean±S.D.) are average from 4 to 6 replicate capillary assays for each duplicate samples in each group, except for group (1) or (2) which are from duplicate or triplicate capillary assays for each of the single sample. Student's t-test was carried out by comparison of group (3) with (4) or (5) with (6). *Non-significant difference is accepted at $p \geq 0.05$.

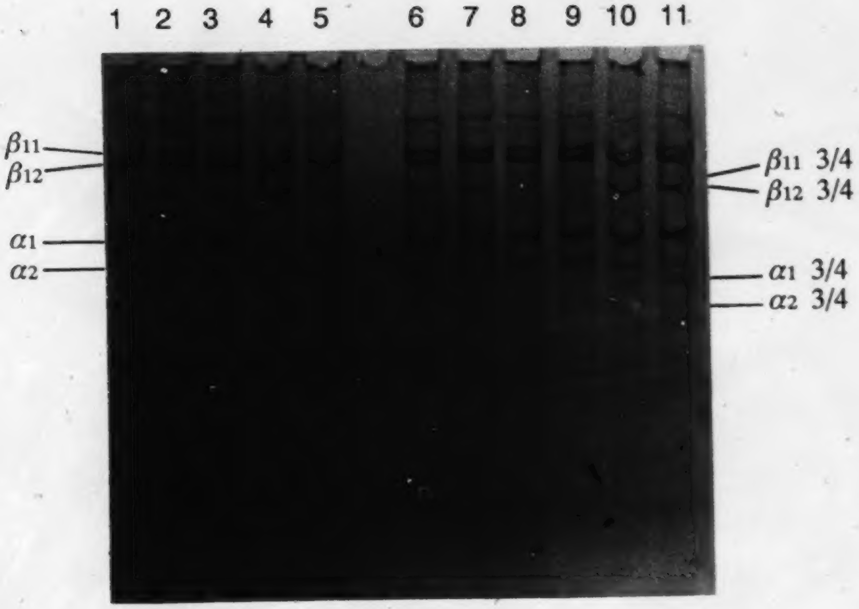


Figure 4.3 Effect of cyclosporine A on hrIL-1α- or hrIL-1ß-induced collagenase production by gingival fibroblasts (CSA-12, passage 5): SDS-PAGE (5%) for collagenase activity assay. Day 2 Samples were concentrated and activated from the culture medium and incubated with collagen for 16 h. at R.T. Lane 1 = no enzyme control; Lane 2 = DMEM-0; Lane 3 = DMEM-0 + CsA 800 ng/mL; Lane 4 and 5 = DMEM-0 + hrIL-1α; Lane 6 and 7 = DMEM-0 + hrIL-1ß; Lane 8 and 9 = DMEM-0 + hrIL-1α + CsA 800 ng/mL; Lane 10 and 11 = DMEM-0 + hrIL-1ß + CsA 800 ng/mL.

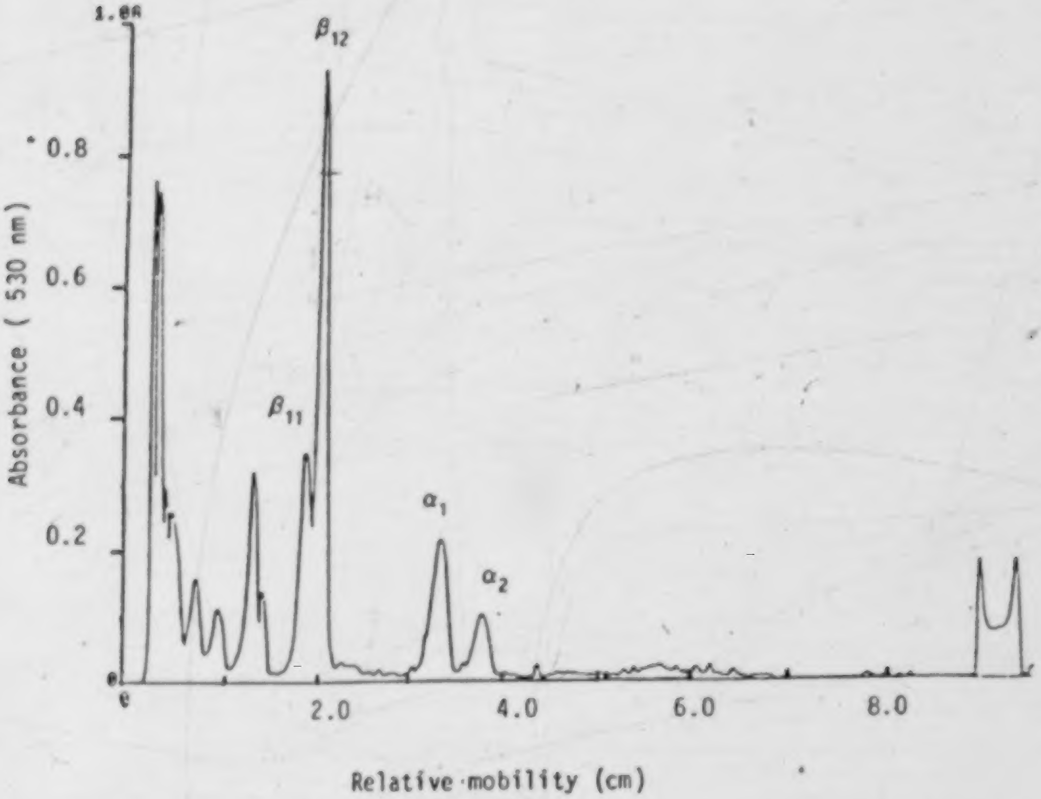
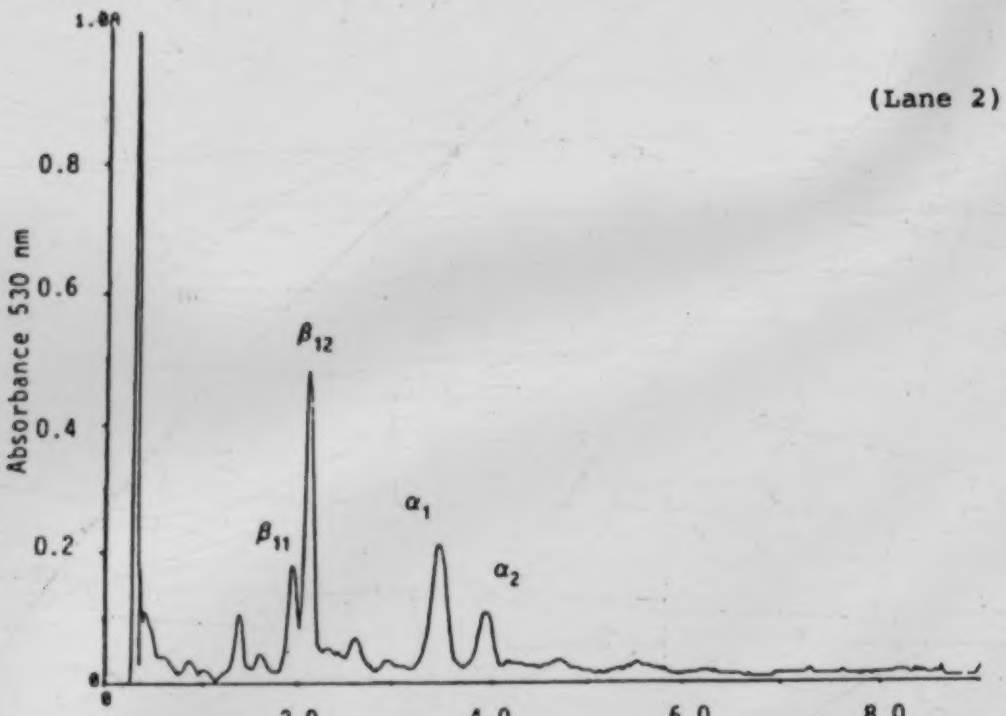


Figure 4.4.a Scanning a 5% SDS-gel for "no enzyme control".

Figure 4.4.b Scanning the gel in Figure 4.3 for lane 2 (DMEM-0) and lane 3 (DMEM-0 + CsA 800 ng/mL). No collagenase activity was seen in these two samples.


1.0A
0.8
0.6
0.4
0.2
0
Absorbance 530 nm
(Lane 2)
β_{12}
β_{11}
α_1
α_2
0
2.0
4.0
6.0
8.0

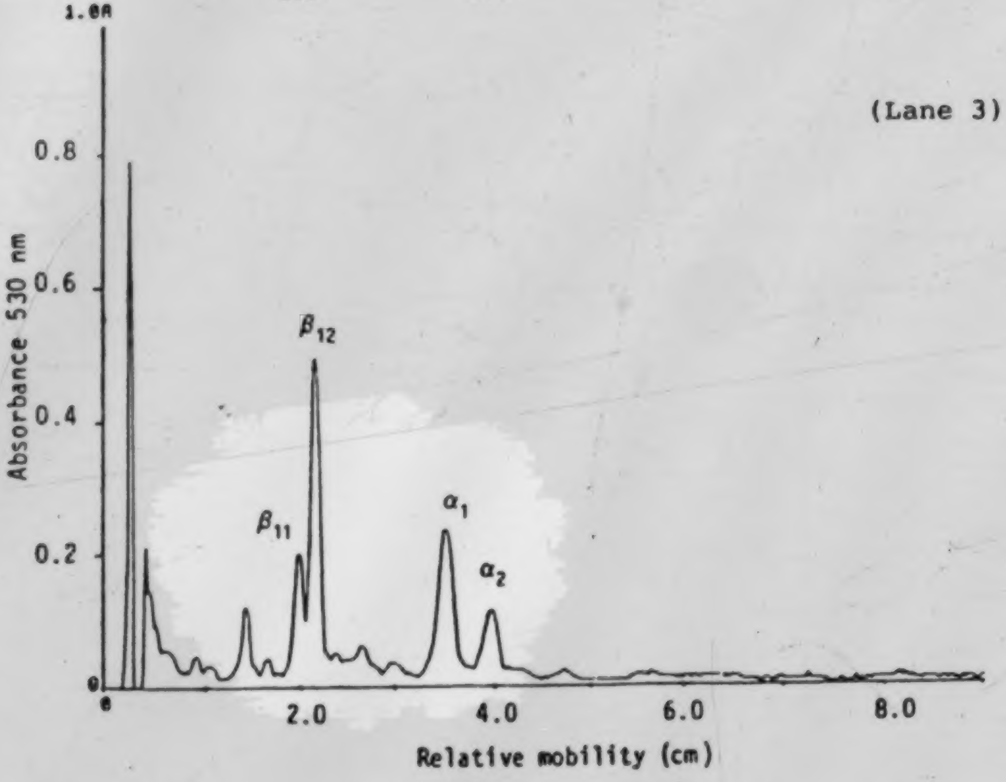

1.0A
0.8
0.6
0.4
0.2
0
Absorbance 530 nm
(Lane 3)
β_{12}
β_{11}
α_1
α_2
0
2.0
4.0
6.0
8.0
Relative mobility (cm)

Figure 4.5 Effect of cyclosporine A on hrIL-1α- or hrIL-1ß-induced collagenase production by gingival fibroblasts (CSA-8, passage 5): SDS-PAGE (5%) for collagenase activity assay. **a.** Day 2 samples; **b.** Day 3 samples. Samples were concentrated and activated from the culture medium and diluted 10-fold before incubation with collagen for 3 h. at R.T. Lane 1 = no enzyme control; Lane 2 = DMEM-0; Lane 3 = DMEM-0 + CsA (800 ng/mL); Lane 4 and 5 = DMEM-0 + hrIL-1α; Lane 6 and 7 = DMEM-0 + hrIL-1ß; Lane 8 and 9 = DMEM-0 + hrIL-1α + CsA (800 ng/mL); Lane 10 and 11 = DMEM-0 + hrIL-1ß + CsA (800 ng/mL).

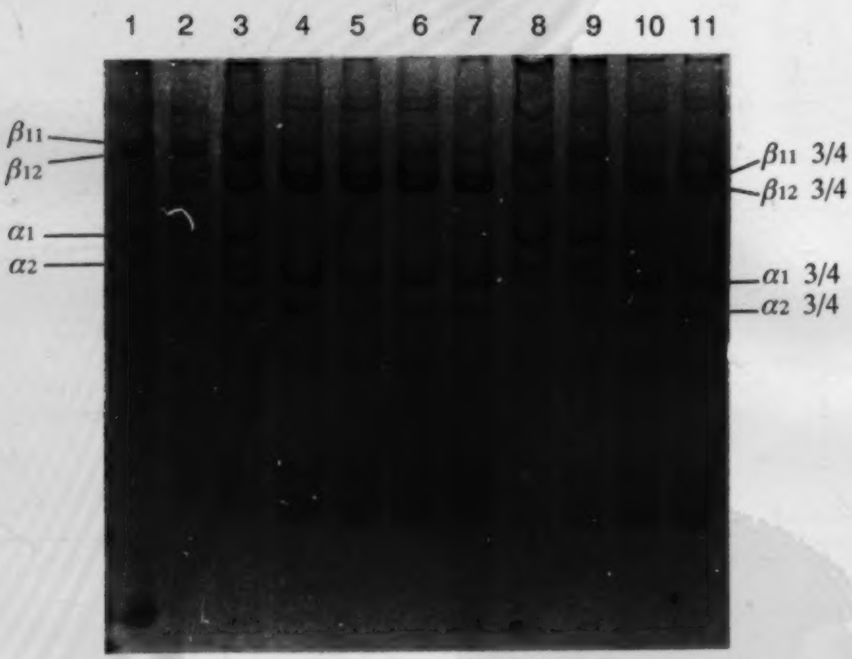


Figure 4.5.a Day 2 samples

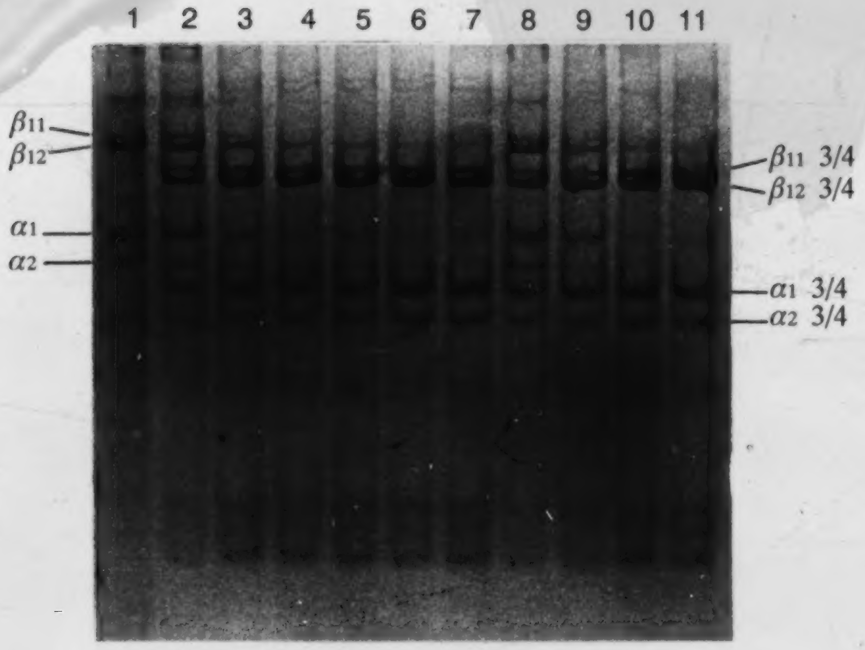
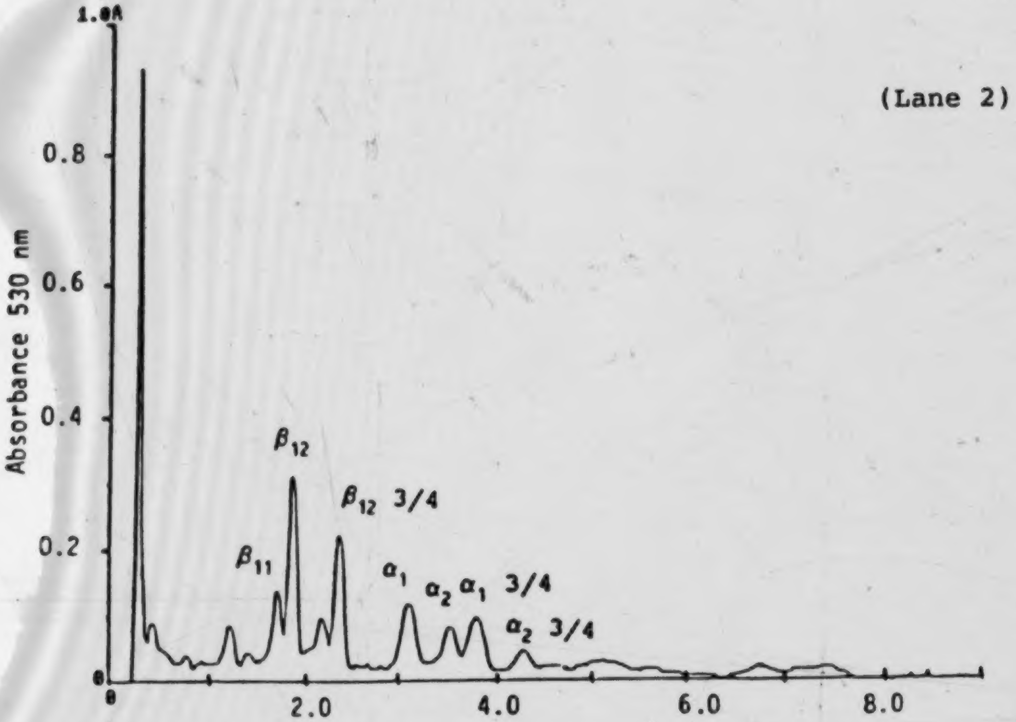


Figure 4.5.b Day 3 samples

Figure 4.6.a Scanning the gel in Figure 4.5.a for lane 2 (DMEM-0) and lane 3 (DMEM-0 + CsA 800 ng/mL). This cell strain had measurable collagenase activity without stimulating agent presence in the culture medium. CsA enhanced the activity of the activated collagenase.


(Lane 2)
1.0A
0.8
0.6
0.4
0.2
0
Absorbance 530 nm
β_{11}
β_{12}
β_{12} 3/4
α_1
α_2
α_1 3/4
α_2 3/4
0
2.0
4.0
6.0
8.0

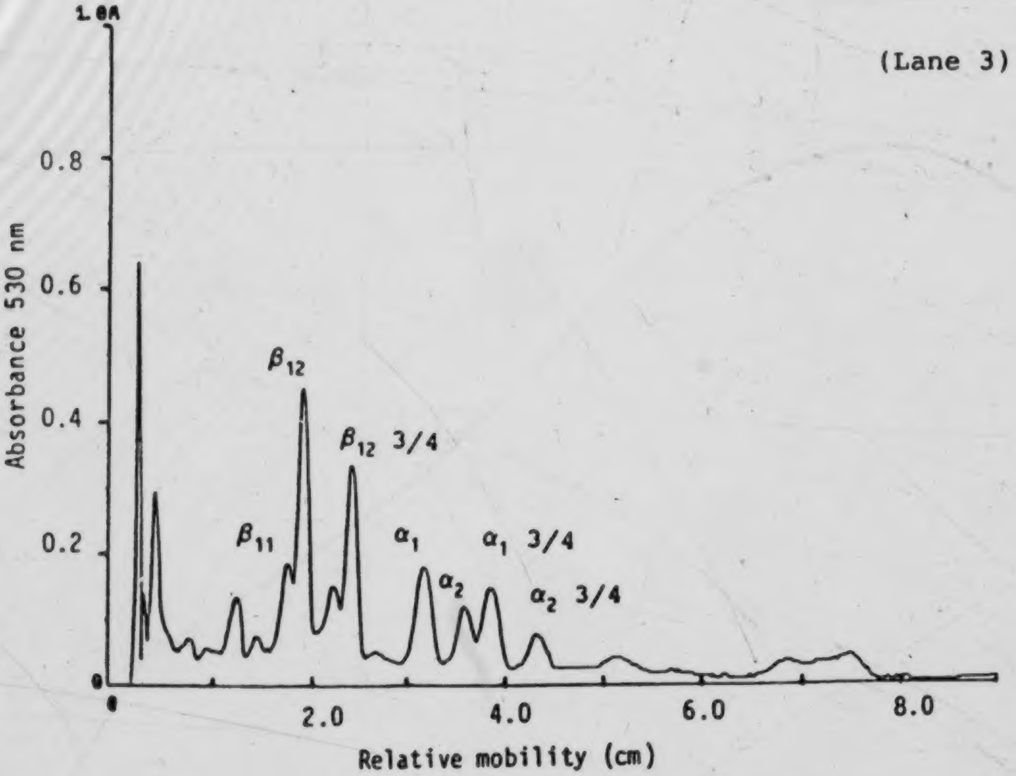

(Lane 3)
1.0A
0.8
0.6
0.4
0.2
0
Absorbance 530 nm
β_{12}
β_{12} 3/4
β_{11}
α_1
α_1 3/4
α_2
α_2 3/4
0
2.0
4.0
6.0
8.0
Relative mobility (cm)

Figure 4.6.b Scanning the gel in Figure 4.5.a for lane 4 (DMEM-0 + hrIL-1α) and lane 9 (DMEM-0 + hrIL-1α + CsA 800 ng/mL). CsA inhibited hrIL-1α-induced collagenase production at about 50% in this 10-fold diluted sample.

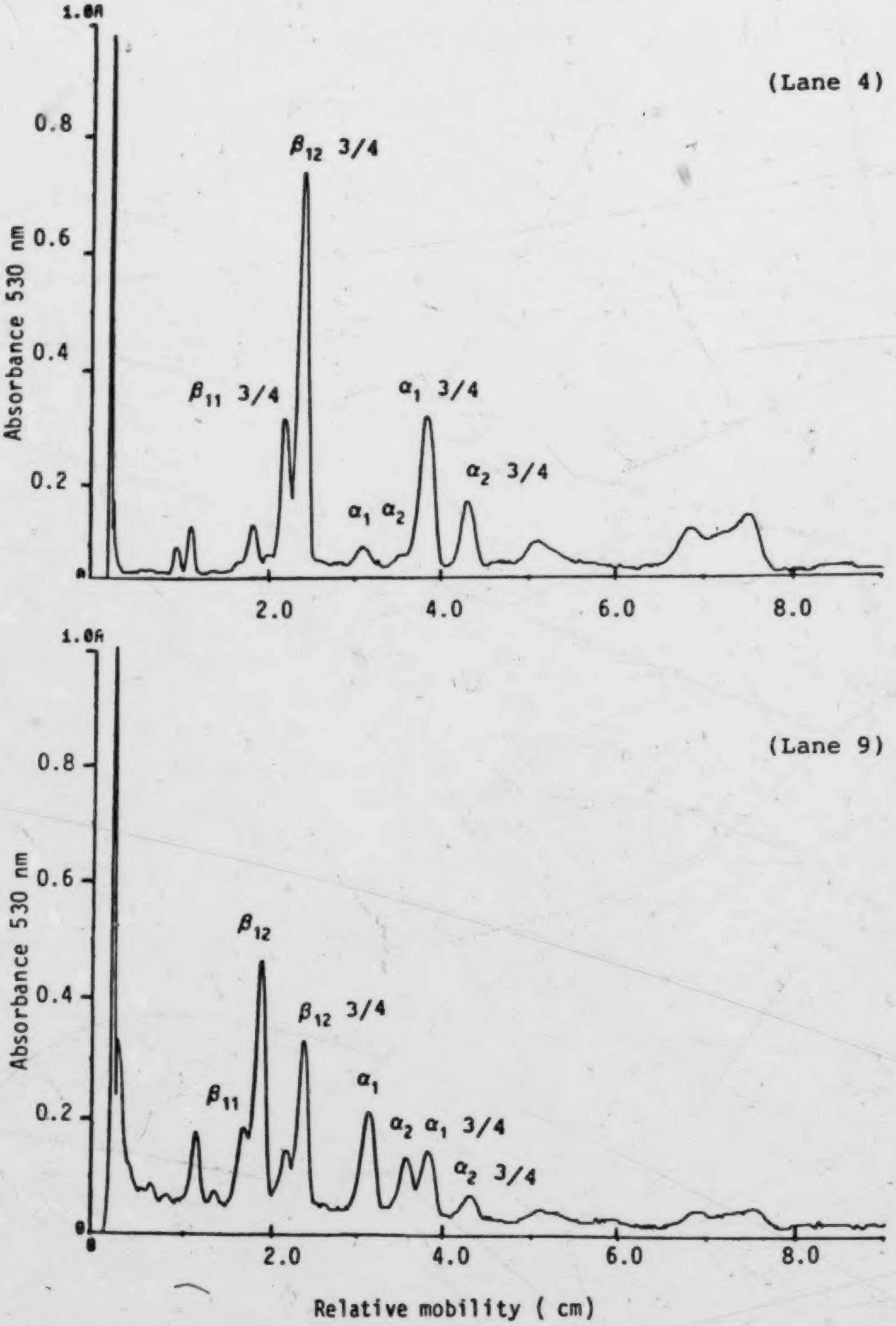
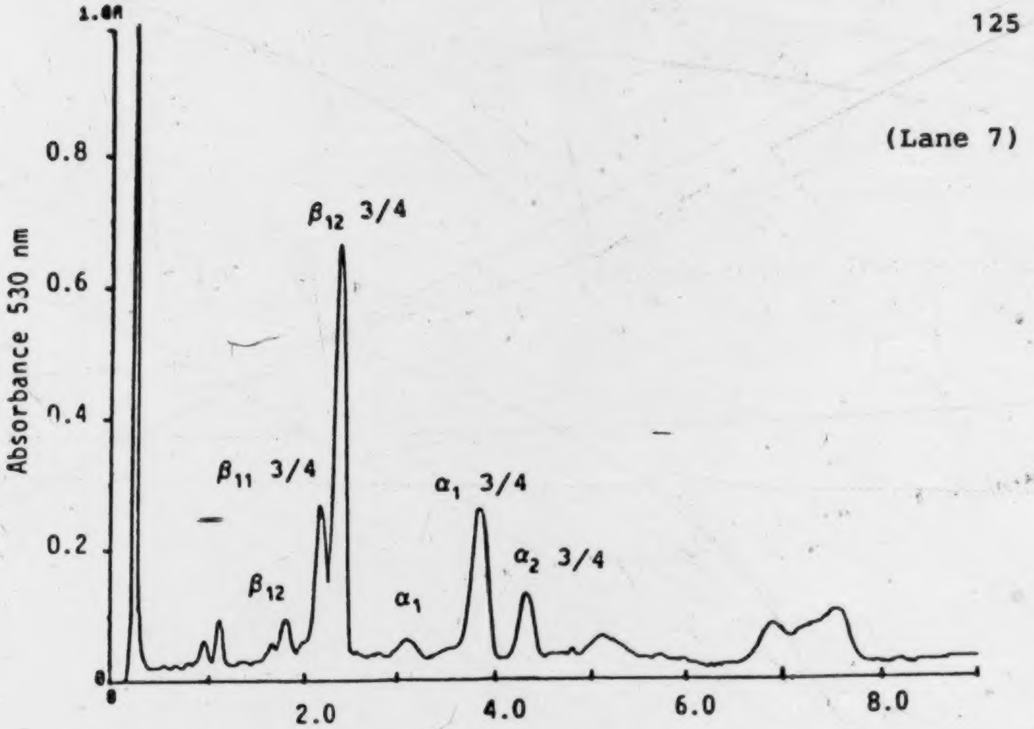
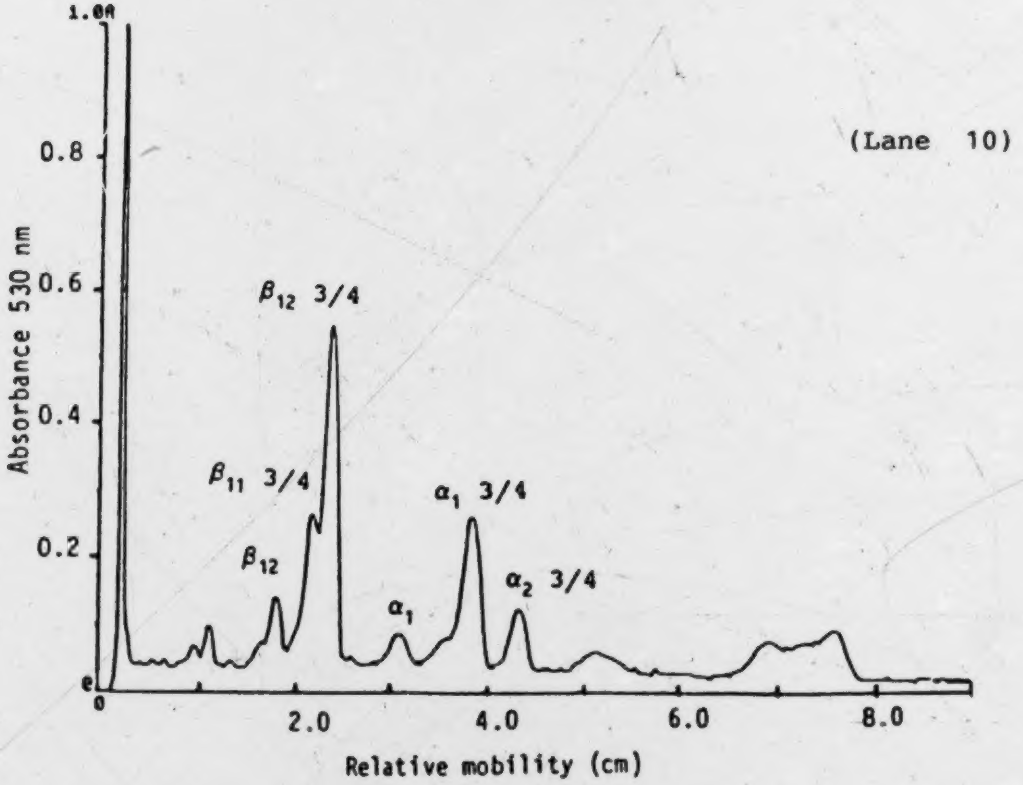

1.0A
(Lane 4)
0.8
0.6
0.4
0.2
0
Absorbance 530 nm
β12 3/4
β11 3/4
α1 3/4
α2 3/4
α1 α2
2.0
4.0
6.0
8.0
1.0A
(Lane 9)
0.8
0.6
0.4
0.2
0
Absorbance 530 nm
β12
β12 3/4
β11
α1
α2 α1 3/4
α2 3/4
0
2.0
4.0
6.0
8.0
Relative mobility (cm)

Figure 4.6.c Scanning the gel in Figure 4.5.a for lane 7 (DMEM-0 + hrIL-1ß) and lane 10 (DMEM-0 + hrIL-1ß + CsA 800 ng/mL). CsA did not affect the effect of hrIL-1ß on stimulating collagenase production by fibroblasts


(Lane 7)
1.0A
0.8
0.6
0.4
0.2
0
Absorbance 530 nm
β_{12} 3/4
β_{11} 3/4
β_{12}
α_1
α_1 3/4
α_2 3/4
0
2.0
4.0
6.0
8.0


(Lane 10)
1.0A
0.8
0.6
0.4
0.2
0
Absorbance 530 nm
β_{12} 3/4
β_{11} 3/4
β_{12}
α_1
α_1 3/4
α_2 3/4
0
2.0
4.0
6.0
8.0
Relative mobility (cm)

4.4 DISCUSSION

It was previously reported that CsA either increased or decreased collagen production (Coley et al., 1986; Tipton and Dabbous, 1986). Our results showed that the fibroblasts were stimulated to produce collagenolytic metalloproteinases in all four experimental systems studied here, i.e., culture on a layer of fibrillar collagen and culture in the presence of ConA, hrIL-1α or hrIL-1ß. This is the first direct evidence that the degradation of fibrillar collagen by fibroblasts is affected by CsA at therapeutic concentrations. Our results are in agreement with those reported by McGaw and Porter (1988) who observed the reduction of frequency of occurrence of intracellular collagen profiles in CsA-induced hyperplastic tissue.

Zebrowski et al (1988) reported that CsA (250 and 500 ng/mL) had no direct effect on collagenase production. This is probably because no agent was added for stimulation of collagenase production in their culture system. In previous work more than 20 cell strains from human gingival fibroblasts have been studied in this laboratory; and it was noticed that in the absence of such stimulation most of these strains secrete very little enzyme (P.G. Scott, unpublished data). In the present study, one exception was seen in CSA-8 (passage 5) in the hrIL-1 experiment (see above). However, this cell strain (also in passage 5) was not seen to produce any collagenase in the non-stimulated group in ConA experiment (see above). It may be that the cells changed their properties of enzyme production either during the process of freezing, storage or thawing. The failure to use any method to stimulate enzyme production may have been responsible for one report (Heath et al., 1983) that gingival fibroblasts did not secrete collagenolytic metalloproteinases and for the inconsistent results of preliminary studies on the effect of CsA on collagenase production in vitro.

The mechanism by which CsA enhanced the activity of activated collagenase (see above) is not understood. It is possibly by way of some physical hydrophobic interaction between CsA and the activated collagenase. Since the enzyme activity from the fibroblast culture medium in the presence of stimulating agents and CsA declined, this suggests that the drug inhibits the production of metalloproteinases in response to stimulating agents rather than simply acting as a collagenase inhibitor. In the presence of CsA the fibroblasts still underwent morphology changes in response to ConA or hrIL-1 treatment, which again suggests that CsA does not act on the membrane of the cells (see Chapter II).

In the experiment on the effect of CsA on ConA-stimulation of collagenase production, one potential error is in the number of the cells. The data in Tables 4.3.a and 4.3.b were not corrected for number of cells, due to the difficulty of trypsinizing the cells off the culture flasks after ConA treatment. This problem was later overcome by quantifying the total DNA by the ethidium fluorescence assay (Morgan et al., 1979) (see chapter V).

Gingival fibroblasts can produce several types of enzyme such as collagenase, gelatinase, stromelysin and telopeptidase (for a review, see Birkedal-Hansen, 1988). The samples prepared for general proteinase and collagenase assays were actually a mixture of enzymes. The collagenase assays used in the present studies are very specific. Although the Azocoll assay is unspecific, it is a simple way to test proteinase activity.

Several case reports have remarked on the association between inflammation and hyperplasia (for a review, see Chapter I) and a positive correlation between severity of gingival overgrowth and gingivitis and plaque indices has been shown (McGaw et al., 1987). Within inflamed gingiva, IL-1 and other mediators of inflammation could increase both biosynthetic and degradative activity of fibroblasts (see Introduction). It is not clear what is the intracellular transduction signal for IL-1α and/or IL-1ß. Ojima et al. (1989) reported an enhancement of IL-1-induced procollagenase production by fibroblasts in co-culture with activated macrophages with the calmodulin inhibitor W-7 [N-6(aminohexyl)-5-chloro-naphthalenesulfonamide]. Georgilis et al. (1987) showed that IL-1ß (100 ng/mL, or 100 half-maximal T cell stimulating units/mL) did not significantly change the intracellular free calcium concentration ([Ca^{2+}]i). We had observed that hrIL-1α (24 half-maximal units/mL) or hrIL-1ß (20 half-maximal units/mL) had no effect on the $[Ca^{2+}]i$ of fibroblasts, as assayed by the Fura 2 method (X. F. Fan and P.G. Scott, unpublished data). After binding to the receptors on diploid fibroblast membranes, IL-1ß was found to be internalized and located in the nucleus after 1 to 4 h. (Qwarnstrom et al., 1988). Whether IL-1α acts in a similar way on fibroblasts is unclear.

It is not understood why CsA selectively inhibits hrIL-1α-but not hrIL-1ß-induced collagenase production in fibroblasts. CsA did not directly act on the IL-1 receptor in T lymphocytes (Tracey et al., 1988). Since there is no difference in IL-1α and IL-1ß receptors on a variety of murine and human cells (Dower and Urdal, 1987), the intracellular pathway for the action of IL-1α and IL-1ß is likely to be the same. However, our results indicate that IL-1α and IL-1ß could possibly have different intracellular mechanisms in fibroblasts. An alternative explanation could be that CsA interacts with IL-1α or IL-1ß extracellularly. The CsA-IL-1α complex could be inactive, while the CsA-IL-1ß is still active. In support of this hypothesis is the fact that the amino acids involved in receptor binding in IL-1α and IL-1ß are different (McDonald et al., 1986; Gronenborn et al., 1988). Alternatively, CsA could selectively bind to hrIL-1α but not to hrIL-1ß in the extracellular solution. This is possible since hrIL-1α is found to be more hydrophobic than hrIL-1ß when the amino acid sequences for these two peptides are compared (P.G. Scott, personal communication).

If collagen synthesis is increased by CsA _per se_ or by an inflammatory mediator such as IL-1 (see above), and the stimulation of collagen degradation _in vivo_ is inhibited concurrently, as it is in these _in vitro_ model systems, it could result in an imbalance in connective tissue metabolism. This may explain, at least in part, the hyperplasia directly caused by the drug and/or complicated by local inflammatory mulation in gingiva. The precise mechanism by which CsA ith collagen breakdown or synthesis is not known.

4.5 BIBLIOGRAPHY

AGGELLER, J., FRISCH, S.M., WERB,Z. Collagenase is a major gene product of induced rabbit synovial fibroblasts. J. Cell Biol. 98:1656-1661, 1984a.

AGGELLER, J., FRISCH, S.M., WERB,Z. Changes in cell shape correlate with collagenase gene expression in rabbit synovial fibroblasts. J. Cell Biol. 98:1662-1671, 1984b.

AURON, P.E., WEBB, A.C., ROSENWASSER, L.J., MUCCI, S.F., RICH, A., WOLFF, S.M., DINARELLO, C.A. Nucleotide sequence of human monocyte interleukin-1 precursor cDNA. Proc. Natl. Acad. Sci. USA. 81:7907-7911, 1984.

BENNETT, W.M., NORMAN, D.J. Action and toxicity of cyclosporine. Ann. Rev. Med. 37:215-224, 1986.

BERMAN, M.B., MANABE, R., DAVISON, P.F. Tissue collagenase: A simplified, semiquantitative enzyme assay. Analyt. Biochem. 54:522-534, 1973.

BIRKEDAL-HANSEN, H. From tadpole collagenase to a family of matrix metalloproteinases. J. Oral Pathol. 17:445-451, 1988.

BISWAS, C., DAYER, J.-M. Stimulation of Collagenase production by collagen in mammalian cell cultures. Cell. 18: 1035-1041, 1979.

BRINCKERHOFF, C.E., McMILLAN, R.M., FAHEY, J.V., HARRIS, E.D., Jr. Collagenase production by synovial fibroblasts treated with phorbol myristate acetate. Arthritis and rheumatism. 22:1109-1116, 1979.

CHAVIRA, R., Jr., BURNETT, T.J. HAGEMAN, J.H. Assaying proteinases with azocoll. Analyt. Biochem. 136:446-450, 1984.

COLEY, C., JARVIS, K., HASSELL, T. Effect of cyclosporine-A on human gingival fibroblasts in vitro. J. Dent. Res. 65 (special issue):353, 1986.

DAYER, J.M. GOLDRING, S.R., ROBINSON, D.R. KRANE, S.M. Cell-cell interactions and collagenase production. In: Collagenase in normal and pathological connective tissues. D.E. Woolley and J.M. Evanson editors. Chichester, UK:Wiley, 1980. pp. 83-104.

DINARELLO, C.A. Interleukin-1. Rev. Infect. Disease. 6:51-95, 1984.

DINARELLO, C.A. Biology of interleukin 1. FASEB. J. 2:108-115, 1988.

DOWER, S.K., KRONHEIM, S.R., HOPP, T.P., CANTRELL, M., DEELEY, M., GILLIS, S., HENNEY, C.S., URDAL, D.L. The cell surface receptors for interleukin-1α and interleukin-1ß are identical. Nature. 324:266-268, 1986.

DOWER, S.K., URDAL, D.L. The interleukin-1 receptor. Immunol. Today. 8:46-51, 1987.

DUNCAN, M.R., BERMAN, B. Differential regulation of collagen, glycosaminoglycan, fibronectin, and collagenase activity production in cultured human adult dermal fibroblasts by interleukin 1-alpha and beta and tumour necrosis factor-alpha and beta. J. Invest. Dermatol. 92:699-706, 1989.

GEORGILIS, K., SCHAEFER, C., DINARELLO, C.A., KLEMPNER, M.S. Human recombinant interleukin 1ß has no effect on intracellular calcium or on functional responses of human neutrophils. J. Immunol. 138:3403-3407, 1987.

GRONENBORN, A.M., WINGFIELD, P, MCDONALD, H.R., SCHMEISSNER, U., CLORE, G.M. Site directed mutants of human interleukin-1α: a ^{1}H-NMR and receptor binding study. FEBS Lett. 231:135-138, 1988.

HARPER, E., BLOCH, K.J., GROSS, J. The zymogen of tadpole collagenase. Biochem. 10:3035-3041, 1971.

HARRIS, E.D., Jr., WELGUS, H.G., KRANE, S.M. Regulation of the mammalian collagenases. Collagen Rel. Res. 4:493-512, 1984.

HEATH, J.K., GOWEN, M., MEIKLE, M.C., REYNOLDS, J.J. Human gingival tissues in culture synthesize three metalloproteinases and a metalloproteinase inhibitor. J. Period. Res. 17:183-190, 1983.

ITO, A., GOSHOWAKI, H., SATO, T., MORI, Y. YAMASHITA, K. HAYAKAWA, T, NAGASE, H. Human recombinant interleukin-1α-mediated stimulation of procollagenase production and supression of biosynthesis of tissue inhibitor of metalloproteinases in rabbit uterine cervical fibroblasts. FEBS letters. 234:326-330, 1988

ITZHAKI, R.F., GILL, D.M. A micro-Biuret method for estimating proteins. Analyt. Biochem. 9:401-410, 1964.

KATSURA, M., ITO, K., HIRAKAWA, S., MORI, Y. Human recombinant interleukin-1α increases biosynthesis of collagenase and hyaluronic acid in cultured human chorionic cells. FEBS lett. 224:315-318, 1989.

LEPE-ZUNIGA, J.L., GERY, I. Production of intra- and extracellular interleukin-1 (IL-1) by human monocytes. Clinical Immunol. Immonopath. 31:222-230, 1984.

MCDONALD, H.R., WINGFIEL, P., SCHMEISSNER, U. SHAW, A., CLORE, G.M., GRONENBORN. Point mutations of human interleukin-1ß with decreased receptor binding affinity. FEBS Lett. 209:295-298, 1986.

MARCH, C.J., MOSLEY, B., LARSEN, A., CERRETTI, D.P., BRAEDT, G., PRICE, V., GILLIS, S., HENNEY, C.S., KRONHEIM, S.R., GRABSTEIN, K., CONLON, P.J., HOPP, T.P., COSMAN: Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature, 315:641-647, 1985.

MATSUSHIMA, K., TAGUCHI, M., KOVACS, E.J., YOUNG, H.A., OPPENHEIM, J.J. Intracellular localization of human monocyte associated interleukin 1 (IL1) activity and release of biologically active IL 1 from monocytes by trypsin and plasmin. J. Immunol. 136:2883-2891, 1986.

MCGAW, W.T., LAM, S., COATES, J. Cyclosporin-induced gingival overgrowth: Correlation with dental plaque scores, gingivitis scores and cyclosporin levels in serum and saliva. Oral Surg. Oral Med. Oral Pathol. 64:293-297, 1987.

MCGAW, W.T., PORTER, H. Cyclosporine-induced gingival overgrowth: An ultrastructural stereological study. Oral Surg. Oral Med Oral Pathol. 65:186-190, 1988.

MECHANIC, G.L. Collagen biochemistry. In: Skeletal Research. Simmons and Kunin editors. Academic Press, NY., 1979. pp. 227-241.

MILLER, E.J. Chemistry of the collagens and their distribution. In: Extracellular matrix biochemistry. K.A. Piez and A.H. Reddi editors. Elsevier, N.Y., 1984. page 62.

MORGAN, A.R., LEE, J.S., PULLEYBLANK, D.E., MURRAY, N.L., EVANS, D.H. Review: Ethidium fluorescence assays. Part I. Physiological studies. Nucleic acids Res. 7:547-569, 1979.

NAKANO, T., SCOTT, P.G. Purification and characterization of gelatinase produced by fibroblasts from human gingiva. Biochem. Cell Biol. 64:387-393, 1985.

OJIMA, Y., ITO, A., NAGASE, H., MORI, Y. Calmodulin regulates the interleukin 1-induced procollagenase production in human uterine cervical fibroblasts. Bioch. Biophys. Acta. 1101:61-66, 1989.

OPPENHEIM, J.J., KOVACS, E.J., MATSUSHIMA, K., DURUM, S.K. There is more than one interleukin 1. Immunol. Today. 7:45-56, 1986.

QWARNSTROM, E.E., PAGE, R.C., GILLIS, S., DOWER, S.K. Binding, internalization, and intracellular localization of interleukin-1ß in human diploid fibroblasts. J. Biol. Chem. 263:8261-8269, 1988.

RICHARDS, D., RUTHERFORD, R.B. The effects of interleukin 1 on collagenolytic activity and prostaglanding-E secretion by human periodontal-ligament and gingival fibroblast. Archs. Oral Biol. 33:237-243, 1988.

SCOTT, P.G., GOLDBERG, H.A. Collagen Rel. Res. Cleavage of the carboxy-terminal cross-linking region of type I collagen by proteolytic activity from cultured porcine gingival explants. 3:295-304, 1983.

SCOTT, P.G., GOLDBERG, H.A., DODD, C.M. A neutral proteinase from human gingival fibroblasts active against the C-terminal cross-linking region of type I collagen. Biochem. Biophys. Res. Communs. 114:1064-1070, 1983.

SELLERS, A., CARTWRIGHT, E., MURPHY, G. REYNOLDS, J.J. Evidence that latent collagenases are enzyme-inhibitor complexes. Biochem. J. 163:303-3307, 1977.

SODEK, J., OVERALL, C.M. Matrix degradation in hard and soft connective tissues. In: The biological mechanism of the tooth eruption and root resorption. Z. Davidovitch editor, EBSCO Media, 1988. pp. 303-311.

STEVEN, F.S., LOWTHER, D.A. Insoluble collagen II. The use of fluorescein labelled polymeric collagen fibrils in a very sensitive assay procedure for enzymes degrading insoluble collagen. Connective Tissue Res. 4:7-10, 1975.

STRICKLIN, G.P., BAUER, E.A., JEFFREY, J.J., EISEN, A.Z. Human skin collagenase: Isolation of precursor and active forms from both fibroblast and organ cultures. Biochem. 16:1607-1614, 1977.

STRICKLIN, G.P., EISEN, A.Z., BAUER, E.A., JEFFREY, J.J. Human skin fibroblast collagenase: Chemical properties of precursor and active forms. Biochem. 17:2331-2337, 1978.

TIPTON, D.A., DABBOUS, M.K. Heterogeneity of gingival fibroblast response to cyclosporine. J. Dent. Res. 65 (Special issue):331, 1986.

TRACEY, D..E., HARDEEE, M.M., RICHARD, K.A., PASLEY, J.W. Pharmacological inhibition of interleukin-1 activity on T cells by hydrocortisone, cyclosporine, prostaglandins, and cyclic nucleotides. Immunopharmacol. 15:47-62, 1988.

WOOLLEY, D.E. Mammalian collagenase. In: Extracellular matrix biochemistry. K. A.Piez and A.H. Reddi editors. Elsevier press, NY., 1984. pp. 119-157.

WYSOCKI, G.P., GRETZINGER, H.A., LAUPACIS, A., ULAN, R. A., STILLER, C.R. Fibrous hyperplasia of the gingiva: A side effect of cyclosporin A therapy. Oral Surg. Oral Med. Oral Pathol. 55:274-278, 1983.

ZEBROWSKI, E.J., RAMAMURTHY, N.S., SINGER, D.L., BRUNKA, J.R. Collagenase activity of cyclosporin, nifedipine and phenytoin treated fibroblasts. J. Dent. Res. _67 (special issue)_:331, 1988..

CHAPTER V

COMPARISON OF SOME OF THE EFFECTS OF NIFEDIPINE AND PHENYTOIN WITH CYCLOSPORINE A ON GINGIVAL FIBROBLASTS *IN VITRO*

5.1 INTRODUCTION

Three different classes of drugs have been frequently reported to cause the side-effect of gingival hyperplasia. They are phenytoin (PHT) (Panuska et al., 1960), nifedipine (NF) (Lederman et al., 1984; Barak et al., 1987) and cyclosporine A (CsA) (see Chapter I). There is a great similarity among these drug-associated gingival hyperplasia in terms of onset, pathology and clinical treatment (for reviews, see Butler et al., 1987 and Chapter I). The molecular mechanisms of their side-effects on gingiva are not understood.

Little is known about the effect of NF on gingival fibroblasts. However, for the PHT-induced gingival hyperplasia (PHT-GH), which has been studied for five decades, there were many reports on the direct effects of PHT on gingival fibroblasts. It was reported that PHT at 5 µg/mL and 10 µg/mL in culture medium increased fibroblast growth (Shafer, 1960; Zebrowski et al., 1988). Both total protein and collagen synthesis were increased about 2-fold by the fibroblasts from PHT-GH tissue compared to fibroblasts from normal individuals

(Hassell et al., 1976). PHT also stimulated normal fibroblasts to synthesize more proteins and collagen if exposed to the drug for a longer period than was required by PHT-GH responder cells (Benveniste and Bitar, 1980). Narayanan et al. (1988) showed that overproduction of collagen in PHT-GH resulted from an increased steady state level of collagen mRNA. Increased accumulation of sulfated glycosaminoglycans, which were mainly in the form of proteoglycans, was also found in the cultures of fibroblasts from PHT-GH (Ballard and Butler, 1974; Kantor and Hassell, 1983). Hassell (1982) reported that collagenase secreted by the fibroblasts from PHT-GH responder was mainly inactive even after brief proteolytic treatment with trypsin.

Zebrowski et al. (1988) cultured human gingival fibroblasts in serum-containing growth medium. They found that collagenase activity was decreased by PHT (5 and 10 μg/mL), increased by NF (0.1 and 0.2 μg/mL), and unaffected by CsA (0.25 and 0.5 μg/mL). All three drugs at the concentrations used increased the DNA contents of the cells.

We had been investigating a variety of biological effects of CsA on gingival fibroblasts in vitro. The major findings of our previous studies were: (1) CsA is not a mitogen for fibroblasts; (2) CsA may not act directly on the cell membranes; (3) CsA can be rapidly concentrated inside the fibroblasts' cytosol; (4) CsA directly suppresses the capacity of gingival fibroblasts to degrade fibrillar collagen; and (5) CsA depresses concanavalin A- (ConA) or human recombinant interleukin-1(α)-induced collagenase production by fibroblasts. However, in cell free collagenase-containing samples, it directly enhances the activity of the activated collagenase (see Chapters II, III and IV for detail). These findings stimulated us to compare some of the effects of PHT and NF with CsA on gingival fibroblasts by using the methods established in previous studies.

The objectives of this study were: to investigate the effects of PHT and NF on the growth rate of gingival fibroblasts; the effects of PHT and NF on the degradation of fibrillar collagen and collagenase production by fibroblasts in vitro. The results were compared with those of CsA described in previous Chapters. Our results indicate that these three drugs may not have the same mode of action on fibroblasts.

5.2 MATERIALS AND METHODS

Cell strains and cell culture materials were the same as in Chapter II. All the cell cultures were done in the Forma CO_2 cell culture incubator which was set up as before (Chapter II). The stock solutions of both PHT (Sigma) and NF (Sigma) were prepared in 95% ethanol at 10 mg/mL and 0.2 mg/mL, respectively. The working solutions of NF (0.2 µg/mL) and PHT (10 µg/mL) were always prepared in the medium (DMEM-10 or

DMEM-0, see below) containing ethanol at the concentration of ≤ 0.1%. Most of the experimental methods used in this paper followed those used in Chapters II and IV.

Briefly, for the measurement of cell growth rate, about 0.5×10^4 cells were seeded into each well (in 4 replicate wells for each treatment) of the 24-well cell culture plates in the presence or absence of PHT (10 µg/mL) or NF (0.2 µg/mL) in DMEM-10 (Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, see Chapter II). The cells were fed every 2 to 3 days and harvested with 0.25% trypsin (Worthington, 1:250) at day 1, 3, 5, 7, 9, 10, 11 and 13, or at day 1, 3, 5, 7, 9, 11, 13 and 14. The cell suspensions were then prepared for counting the number of cells with a Coulter counter (Model ZM) as described before (Chapter II). For fibroblasts degrading the FITC- (fluorescein isothiocyanate) labelled fibrillar collagen, the FITC-collagen-coated wells in the 24-well plates had been incubated with PBS (phosphate buffered-saline) for 7 days with daily changes of fresh PBS to reduce the auto-released fluorescence to a suitable level. Fibroblasts were then seeded at confluent density (about 2.4 to 2.5×10^5 cells/well) and incubated in DMEM-10 for 4 days. At day 1, no drug was added into the medium, but at day 2, 3 and 4, PHT (10 µg/mL), NF (0.2 µg/mL) or CsA (0.8 µg/mL) was added into the culture medium, with 3 to 6 replicate wells for each group (see below). The medium in the wells was collected every day and the cells were refed with DMEM-10 with or without NF, PHT or CsA. The fluorescence in the culture medium was measured with a Perkin-Elmer LS-5 luminescence spectrophotometer as in previous experiments (Chapter IV). The cells were harvested by 0.25% trypsin (1:250) and 25 units of bacterial collagenase (Worthington, CLSPA) as before (Chapter IV). In order to study the effect of NF or PHT on collagenase production by fibroblasts, cells had been grown in DMEM-10 to confluence in the 75 cm^2 flasks in about 2 weeks. The cells were incubated in DMEM-0 (serum free medium supplemented with 5% lactalbumin hydrolysate, from DIFCO Laboratories) (12 mL/flask) with or without 25 µg/mL ConA (Pharmacia), 10 µg/mL PHT or 0.2 µg/mL NF for three days. The media were collected each day and the fresh medium added to the flasks. The samples were concentrated with ammonium sulfate (Schwarz/Mann Biotech, Ultra Pure™), then activated by trypsin (Worthington, TPCK). Soybean trypsin inhibitor was added to terminate the action of trypsin, as described in Chapter IV. These concentrated (about 10-fold) and activated samples were in Tris-HCl buffer-1 (0.2 M-KCl/0.005 M-$CaCl_2$/0.02%, w/v, sodium azide/0.05 M Tris-HCl, pH 7.4). The acid soluble collagen (ASC) prepared from bovine skin in our laboratory (E. Edwards) and the stock solution was 2 mg/mL in 0.01 M acetic acid (Chapter IV). The working solution of ASC was 1 mg/mL neutralized by 2-fold concentrated Tris-HCl buffer-1, pH 7.4 (Chapter IV) and used as collagenase substrate. The samples (50 µL), with or without further dilution with Tris-HCl buffer-1, were incubated with

50 µL of the enzyme substrate at room temperature (R.T) for 2 to 16 h., depending on the enzyme activity in the samples. After terminating the incubation, the above enzyme and substrate (digested and/or denatured) mixtures were prepared for running on 5% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) for collagenase activity assays (Chapter IV). The M.W. standard, as well as a no-enzyme control were added onto the gels for SDS-PAGE (Chapter IV). The collagen added to the gel was about 6 or 12 µg/lane.

In order to overcome the problem of correcting the collagenase activity with the cell number in the ConA-treated fibroblasts, the total DNA concentrations of the cells in a culture flask were quantified by the ethidium bromide (Sigma) fluorescence assay (Morgan et al., 1979). At day 3 of the experiment we attempted to trypsinize fibroblasts off the flasks but most of the ConA-treated fibroblasts were not released. The cells were then soaked in 4 mL of PBS for freezing (at -20°C) and thawing (at R.T.) each for 2 h. and three times, respectively. The solutions which contained the soluble components (including DNA) of the cells were collected and pooled with the trypsinized fraction of the cells (about 8 mL per sample in total volume). The samples were extracted with 20 mL of 95% ethanol twice to remove lipids. The precipitates were recovered by centrifugation at 8000 rpm, 4°C for 30 mim. The sediments were then treated with 5 mL of 10% cold TCA (trichloroacetic acid, Fisher) twice to remove the acid soluble contents and the precipitates were recovered as above (Schneider, 1957). The samples were redissolved in 5 mL of Tris-HCl buffer-2 (5 mM-Tris-HCl/0.5 mM EDTA, pH 8.0). Usually the samples were not completely dissolved in the buffer because of the impurity of the DNA, but this is not expected to affect the ethidium bromide assay.

The DNA-containing sample (0.05 mL, in duplicate) was mixed with 2.45 mL ethidium bromide-containing buffer (0.5 µg/mL of ethidium bromide in Tris-HCl buffer-2, pH 8.0) in a polystyrene cuvette (1x1 cm). The fluorescence levels were measured in the Perkin Elmer LS-5 luminescence spectrophotometer with excitation wavelength 475 nm (slit width = 5 nm) and emission wavelength 595 nm (slit width = 20 nm). The instrument was zeroed with the control (0.05 mL Tris-HCl buffer-2 in 2.45 mL ethidium bromide-containing buffer). The calibration curve for DNA (at concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 µg/mL) was prepared in a similar way in duplicate with purified bovine spleen DNA (from Dr. R.A. Morgan, Dept. of Biochemistry, University of Alberta). The DNA standard was about 90% pure as determined by spectrophotometry (Varian, Model DMS 200) with OD_{260nm} /OD_{280nm} = 1.65 to 1.69, whereas, 100% pure DNA should have OD_{260nm}/OD_{280nm} = 1.8 (Maniatis et al., 1982).

The [DNA] calibration curve was linear over the concentration range of 0.2 to 1.0 µg/mL (data not shown). The DNA in the samples (5 mL/flask cells) was measured at 50-fold dilution. The [DNA] is presented as [DNA] µg/flask.

The bands of the denatured and/or digested collagen resolved in the 5% SDS-PAGE gels were scanned with the Joyce-Loebel Scanner at 530nm. The peak areas of α_1 and α_1 3/4 fragments were measured with a digitizer (Model MOP-3, Carl Zeiss Inc.). The percentage of collagen digestion was calculated using the following equation:

$$\%\text{ of digested collagen} = \frac{(\alpha_1\ 3/4)\times 4/3}{(\alpha_1\ 3/4)\times 4/3+(\alpha_1)} \times 100\%$$

Where α_1 and (α_1 3/4) are the peak areas (mm^2) in the scans.

This percentage of digested collagen per sample was then converted into percentage of digested collagen per 100 µg DNA in a sample.

5.3 RESULTS

5.3.1 Effects of nifedipine and phenytoin on gingival fibroblast proliferation rate.

Effects of NF or PHT on fibroblast proliferation were measured with four cell strains, i.e., normal cell strains: N1106 and HUGE-15 and CsA-GH responder cell strains: CSA-8, CSA-12, passage 3, 4, 6 and 5, respectively. The number of cells in each day (except day 0, the day of seeding cells) are average of 4 replicate wells counting 2 or 4 times each. While at day 0, the cell suspensions prepared for seeding into the wells were counted 4 times in each sample, except for HUGE-15 which was not counted at day 0.

The third order polynomial regressions for each growth curve (i.e., "no drug", "NF" and "PHT") and "whole regression" (average data for all three treatments) in two cell strains are shown in Figures 5.1.a and 5.1.b. All of the three individual regression curves in each of the four experiments had coefficient values (R-value) >0.97. They were so close to each other that even the "whole regression" curve also gave a high correlation coefficient (R >0.98). It was therefore concluded that the two drugs had no effect on fibroblast proliferation. These results were similar to those with CsA (Chapter II).

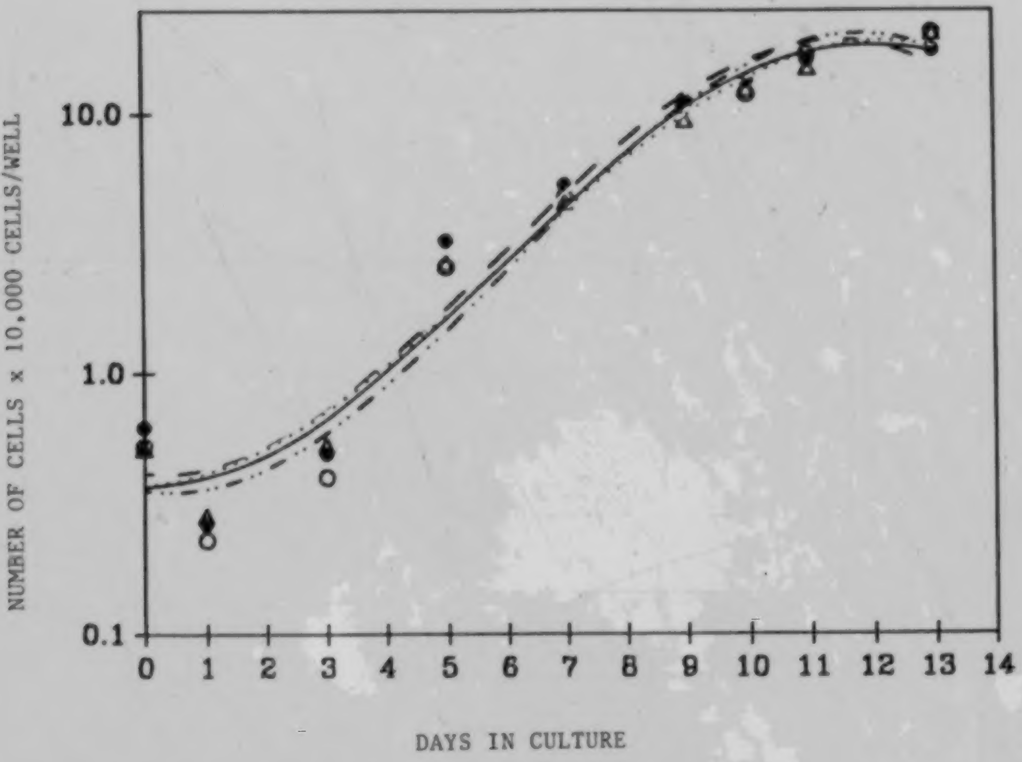


Figure 5.1.a Effect of nifedipine or phenytoin on fibroblast (HUGE-15, passage 4) proliferation. Third order polynomial regressions for the growth curves were: (o-•-•-•-o-•-•-•-o) No drug (R=0.998); (•-----•-----•) [NF] 0.2 μg/mL (R=0.999); (Δ....Δ....Δ) [PHT] 10 μg/mL (R=0.998); (————) "Whole regression" (R=0.997).

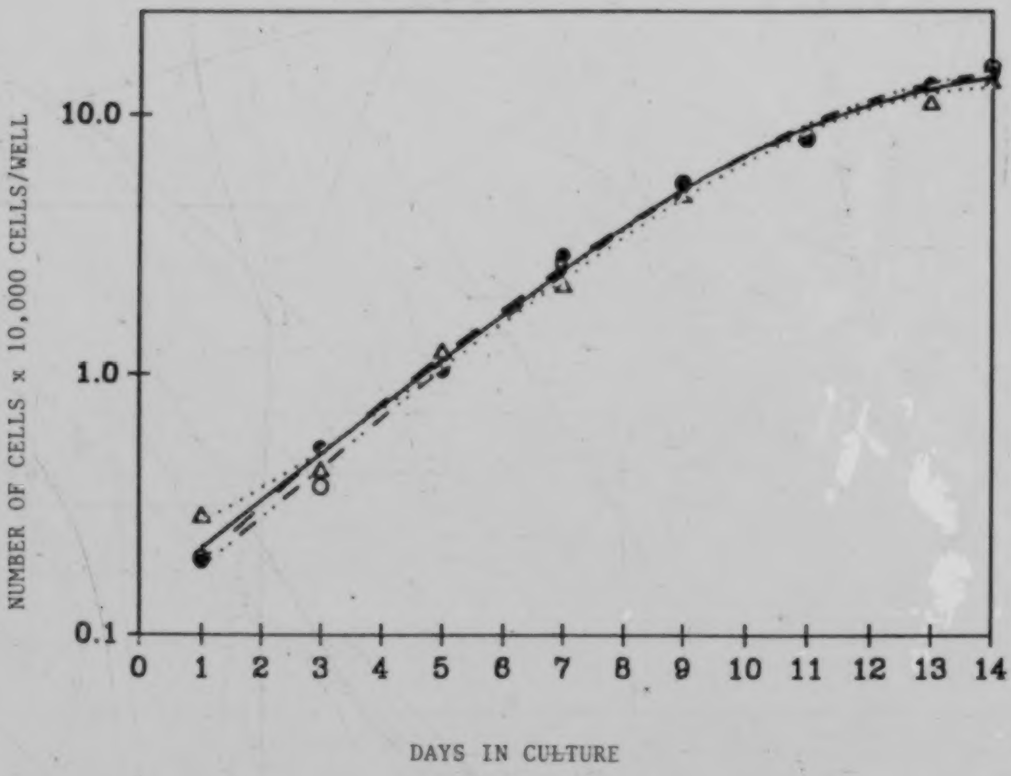


Figure 5.1.b Effect of nifedipine or phenytoin on fibroblast (CSA-8, passage 6) proliferation. Third order polynomial regressions for the growth curves were: (o-··-··-o-··-··-o) No drug (R=0.981); (•-----•-----•) [NF] 0.2 μg/mL (R=0.978); (Δ....Δ....Δ) [PHT] 10 μg/mL (R=0.986); (————) "Whole regression" (R=0.980).

5.3.2 Comparison of the effects of nifedipine and phenytoin with cyclosporine A on degradation of fibrillar collagen by fibroblasts.

Unlike CsA, which strongly depressed the degradation of fibrillar collagen by gingival fibroblasts in all of the cell strains tested (Tables 5.1.a, 5.1.b and Chapter IV), NF and PHT had no effects on the fibroblasts from two CsA-GH responder cell strains (Table 5.1.b). Among three normal cell strains tested, NF had a strong suppression effect on N1106, a weak effect on HUGE-12 and no effect on HUGE-15 (Table 5.1.a). PHT also strongly inhibited the degradation of collagen fibrils by N1106, but in HUGE-12 and HUGE-15, this effect was weak (Table 5.1.a). The suppression of the breakdown of fibrillar collagen by fibroblasts was strongest by CsA (Table 5.1.a).

Table 5.1 Comparison of the effects of nifedipine and phenytoin with cyclosporine A on digestion of fibrillar collagen by gingival fibroblasts.

a. Normal cell strains.

Cell strain (passage)	Drug added	Accumulated Fluorescence	Cellsx10^5 per well	Fluorescence per $1x10^5$ cells
N1106	None	500.4±18.7	0.76±0.1	663.3±32.8
(7)	[NF]	391.5±18.7	0.75±0.1	535.2±57.7 ($p<0.001$)
	[PHT]	373.3±33.2	0.86±0.1	444.5±48.7 ($p<0.001$)
	[CsA]	278.76±7.6	0.75±0.1	373.4±29.8 ($p<0.001$)
HUGE-12	None	582.9±35.6	0.97±0.3	559.9±34.2
(4)	[NF]	455.8±52.8	1.00±0.2	452.2±52.4 ($p=0.041$)
	[PHT]	451.3± 7.7	0.89±0.2	502.4± 8.2 ($p=0.047$)
	[CsA]	370.4±15.9	1.05±0.2	356.5±15.4 ($p=0.001$)
HUGE-15	None	399.3±52.6	1.32±0.2	302.5±39.9
(3)	[NF]	352.2±28.1	1.28±0.2	275.8±22.0 ($p=0.280^*$)
	[PHT]	290.6±60.4	1.29±0.1	226.0±47.0 ($p=0.048$)
	[CsA]	266.7±35.2	1.21±0.2	220.2±29.0 ($p=0.016$)

Table 5.1 Comparison of the effects of nifedipine and phenytoin with cyclosporine A on digestion of fibrillar collagen by gingival fibroblasts.

b. CsA-GH responder cell strains.

Cell strain (passage)	Drug added	Accumulated Fluorescence	Cellsx10^5 per well	Fluorescence per $1x10^5$ cells
CSA-12	None	201.7±15.7	0.95±0.2	210.3±16.6
(4)	[NF]	199.2±14.3	0.86±0.1	231.1±16.6 (p=0.150*)
	[PHT]	217.0±22.9	0.89±0.2	241.1±25.4 (p=0.110)
	[CsA]	143.1±21.7	0.89±0.1	160.80±24.3 (p=0.013)
CSA-8	None	259.8±12.0	1.49±0.2	190.4±15.5
(6)	[NF]	266.2±19.4	1.29±0.3	223.5±40.0 (p=0.510*)
	[PHT]	259.1±42.4	1.37±0.4	214.7±59.1 (p=0.240*)
	[CsA]	204.7±13.3	1.43±0.3	145.2±19.4 (p<0.000)

The data for N1106, HUGE-12, HUGE-15, CSA-12 and CSA-8 are average of 8, 3, 4, 4 and 6 replicate wells, respectively. The fluorescence levels are presented as 4 days' accumulated fluorescence (Mean±S.D.), with or without correction by cell number. Student's t-test was done by comparison of each of the "drug-containing group" with the "no drug" one. *Non-statistical difference is accepted at p≥0.05.

5.3.3 Effects of nifedipine and phenytoin on collagenase activity in the fibroblast culture medium.

Three cell strains were used to investigate the effect of NF (0.2 μg/mL) or PHT (10 μg/mL) on ConA-induced collagenase production by fibroblasts. The results were not consistent.

In CSA-8 (passage 5), at day 1, all the activated samples had slight collagenase activities and ConA did not enhance the enzyme production in most of the groups except in the presence of PHT data not shown). Since the enzyme activities were very strong in the samples both at day 2 and day 3, the collagenase substrate was incubated with 10-fold diluted samples at R.T for 2 h. and the digestion was measured by the 5% SDS-PAGE. The gels of Figures 5.2.a and 5.2.b were scanned and the peak areas of the α_1 and α_1 3/4 fragments were measured as described in Materials and Methods. The results were presented in Table 5.2.

The cells produced measurable collagenase activities in the non-stimulated group (i.e., culture in DMEM-0 without ConA, NF or PHT) in all 2 and 3 days' cultures. At day 2 and 3, NF or PHT suppressed this "auto-release" of collagenase activity (Figures 5.2.a and 5.2.b). However, ConA overcame this suppression effect of NF or PHT (Table 5.2).

Figure 5.2. Effect of nifedipine or phenytoin on concanavalin A-stimulated collagenase production by gingival fibroblasts (CSA-8, passage 5): SDS-PAGE (5%) for collagenase activity assay. **a.** Day 2 samples; **b.** Day 3 samples. Lane 1 = no enzyme control; Lane 2 and 3 = DMEM-0 (no drug); Lane 4 = DMEM-0 + NF (0.2 μg/mL); Lane 5 = DMEM-0+ PHT (10 μg/mL); Lane 6 and 7 = DMEM-0 + ConA (25 μg/mL); Lane 8 and 9 = DMEM-0 + ConA (25 μg/mL) + NF (0.2 μg/mL); Lane 10 and 11 = DMEM-0 + ConA (25 μg/mL) + PHT (10 μg/mL).

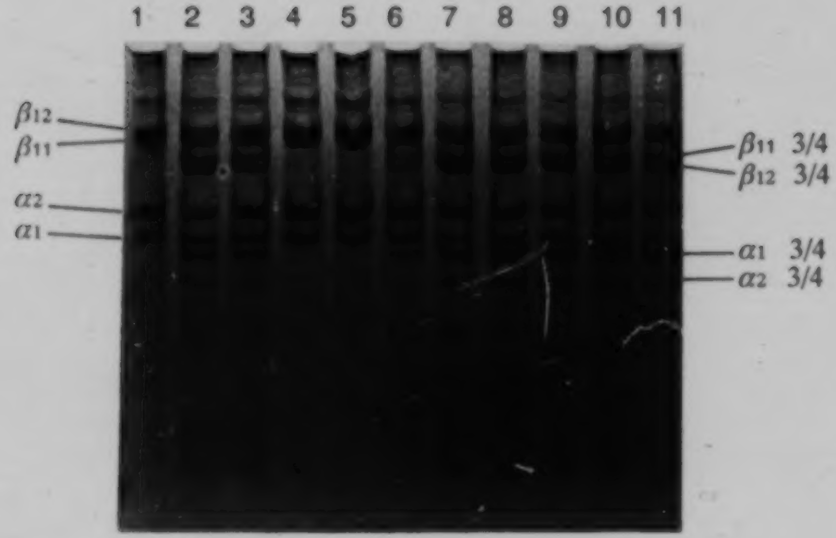


Figure 5.2.a Day 2 samples

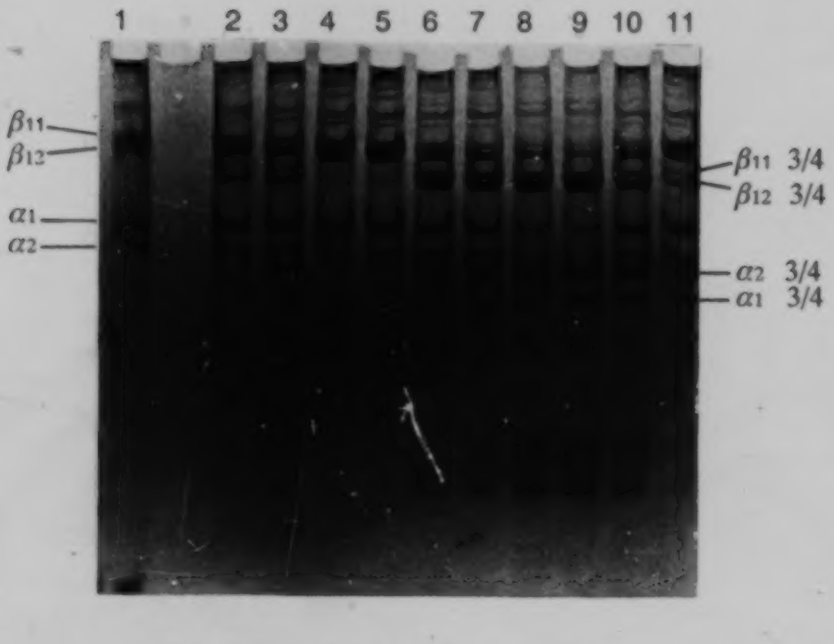


Figure 5.2.b. Day 3 samples

Table 5.2.a Effects of nifedipine or phenytoin on collagenase activity in fibroblast medium. (Day 2 samples of CSA-8, passage 5).

Sample (DMEM-0)	Peak areas (mm^2) α_1	α_1 3/4	[DNA] (µg/sample)	% of collagen digested/100 µg DNA
No drug	177.1±30.6	81.0± 6.4	45.6± 4.4	15.9± 6.6
+NF	279.4	0.0	41.1±12.9	0.0
+PHT	237.7	0.0	51.1± 3.4	0.0
+ConA	176.7±14.6	89.9±50.3	56.4±16.2	19.7±18.8
+ConA+NF	152.9± 5.7	100.0± 2.0	48.8± 1.4	23.9± 0.7 ($p=0.710^*$)
+ConA+PHT	133.9± 9.8	103.6±26.2	69.2±20.2	18.6± 2.9 ($p=0.920^*$)

Table 5.2.b Effect of nifedipine or phenytoin on collagenase activity in fibroblast medium. (Day 3 samples of CSA-8, passage 5).

Sample	Peak are (mm^2)		[DNA]	% of collagen
(DMEM-0)	$\underline{\alpha_1}$	$\underline{\alpha_1\ 3/4}$	(μg/sample)	digested/100 μg DNA
No drug	154.9± 8.1	47.4±22.1	45.6± 4.4	12.4± 9.1
+NF	268.6	0.0	41.1±12.9	0.0
+PHT	227.7	0.0	51.1± 3.4	0.0
+ConA	119.0± 4.5	162.9±62.0	56.4±16.2	30.8±25.1
+ConA+NF	60.8± 4.7	177.8±14.8	48.8± 1.4	40.9± 1.5 (p=0.440*)
+ConA+PHT	140.4±37.1	87.2±51.5	69.2±20.2	17.8±14.8 (p=0.450*)

The data are from duplicate flasks for all "peak areas" (Mean± S.D.), except "+NF" and "+PHT" which had a single test for each. The [DNA] are average of 2 to 4 replicate assays. In the Student's t-test of "+ConA" with "+ConA+NF' or "+ConA+PHT", *non-statistical difference is accepted at $p \geq 0.05$.

In CSA-12 (passage 4), no dilution of the samples of day 1 and day 2 for the collagenase activity assay was required. No collagenase activity was found in any samples at day 1 (data not shown) or in any non-ConA-treated group. However, at day 3 the collagenase activities in ConA-treated groups with or without the presence of NF or PHT were too strong to see any differences (data not shown). Only at day 2, the results showed that NF decreased the ConA-induced collagenase production (Figure 5.3). The lack of a statistically significant effect of PHT may be ascribed to the very high variability encounted in the DNA assay of these cell cultures (Table 5.3).

In N1106 (passage 4), no dilution of the samples of day 1 and day 2 was required. No "auto-releases" of collagenase activity was detected in any sample. At day 1, the cells did not respond to ConA-stimulation (data not shown). At day 2 and 3, this stimulation was remarkable. At day 2, NF suppressed whilst PHT enhanced the ConA effects on the cells (Figure 5.4 and Table 5.4). At day 3, the enzyme activities were too strong to see any differences between the groups (data not shown).

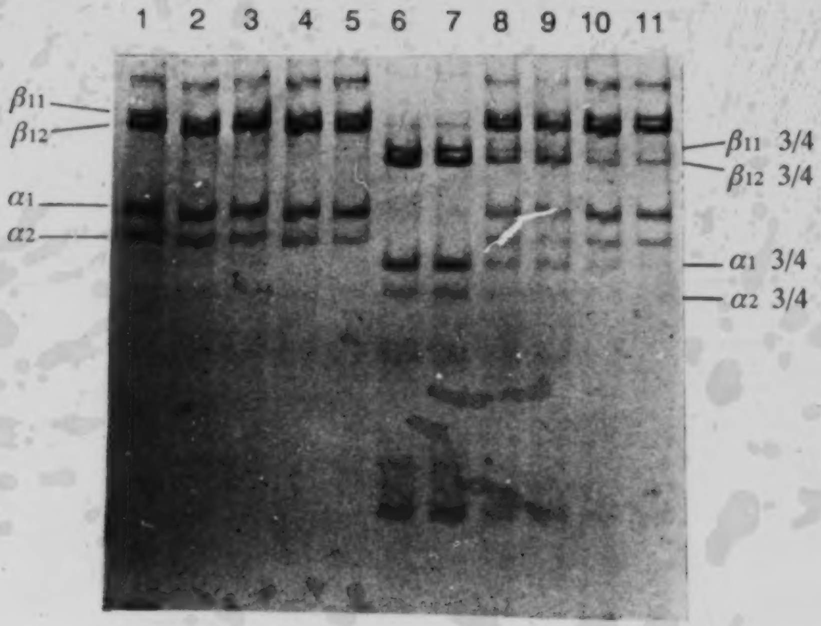


Figure 5.3 Effect of nifedipine or phenytoin on concanavalin A-stimulated collagenase production by gingival fibroblasts (CSA-12, passage 4): SDS-PAGE (5%) for the collagenase activity assay. The concentrated and activated day 2 samples were not diluted before incubation with the collagen at R.T. for 16 h. Lane 1 = no enzyme control; Lane 2 and 3 = DMEM-0 (no drug); Lane 4 = DMEM-0 + NF (0.2 μg/mL); Lane 5 = DMEM-0 + PHT (10 μg/mL); Lane 6 and 7 = DMEM-0 + ConA (25 μg/mL); Lane 8 and 9 = DMEM-0 + ConA (25 μg/mL) + NF (0.2 μg/mL); Lane 10 and 11 = DMEM-0 + ConA (25 μg/mL) + PHT (10 μg/mL).

Table 5.3 Effects of nifedipine and phenytoin on collagenase activity in fibroblast medium. (Day 2 samples of CSA-12, passage 4).

Sample	Peak areas (mm^2)		[DNA]	% of collagen
(DMEM-0)	α_1	α_1 3/4	(µg/sample)	digested/100 µg DNA
No drug	64.3±11.6	0.0	38.4±2.5	0.0
+NF	66.9	0.0	55.0±2.8	0.0
+PHT	51.9	0.0	47.0±6.7	0.0
+ConA	7.5±10.5	90.3±0.6	36.5±2.6	64.8±6.0
+ConA+NF	42.8±0.3	30.4±3.3	39.1±2.5	31.1± 1.5 (p=0.016)
+ConA+PHT	53.0±9.8	17.0±8.2	77.8±44.5	37.0±24.3 (p=0.260*)

The gel in Figure 5.3 were scanned and the area of two peaks were measured. The data are average of duplicate flasks, except "+NF" and "+PHT" which had single test, respectively. The [DNA] are average of 2 to 4 replicate assays (Mean±S.D.). *Non-statistical difference is accepted at p≥0.05.

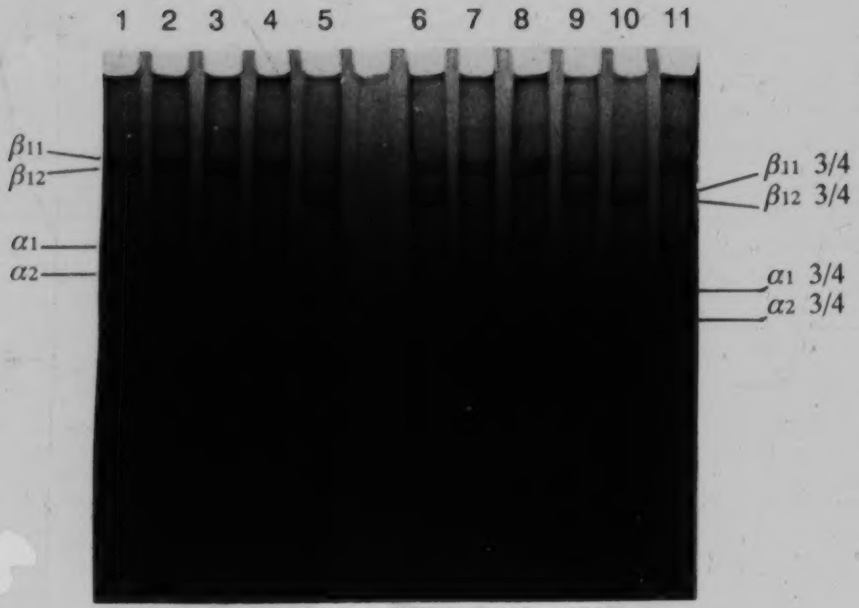


Figure 5.4 Effect of nifedipine or phenytoin on concanavalin A-stimulated collagenase production by gingival fibroblasts (N1106, passage 4): SDS-PAGE (5%) for the collagenase activity assay. The concentrated and activated day 2 samples were not diluted before incubation with the collagen at R.T. for 16 h. Lane 11 = no enzyme control; Lane 1 and 2 = DMEM-0 (no drug); Lane 3 = DMEM-0 + NF (0.2 µg/mL); Lane 4 = DMEM-0 + PHT (10 µg/mL); Lane 5 and 6 = DMEM-0 + ConA (25 µg/mL); Lane 7 and 8 = DMEM-0 + ConA (25 µg/mL) + NF (0.2 µg/mL); Lane 9 and 10 = DMEM-0 + ConA (25 µg/mL) + PHT (10 µg/mL).

Table 5.4 Effects of nifedipine and phenytoin on collagenase activity in fibroblast medium. (Day 2 samples from N1106, passage 4).

Sample (DMEM-0)	Peak areas (mm^2) $\underline{\alpha_1}$	 $\underline{\alpha_1\ 3/4}$	[DNA] (μg/sample)	% of collagen digestion/100 μg DNA
No drug	36.3±13.7	0.0	102.1± 5.1	0.0
+NF	76.1	0.0	(loss)	0.0
+PHT	69.0	0.0	62.9±15.0	0.0
+ConA	22.2±7.9	42.9±0.1	55.4±11.0	33.1±3.8
+ConA+NF	57.6±6.9	16.1±1.8	54.2±10.0	12.9±4.6 ($p=0.040$)
+ConA+PHT	41.9	62.0	29.5± 6.0	48.5 (No statistics*)

The gel of Figure 5.4 were scanned and two of the peak areas were measured. The data are average of duplicate flasks, except "DMEM-0+NF" and "DMEM-0+PHT" which had a single test, respectively. The [DNA] are average of 2 to 4 replicate assays (Mean±S.D.). On comparison of "+ConA" with "+ConA+NF" by Student's t-test, NF significantly inhibited ConA effect on the cells ($p<0.05$). *No statistics for this sample due to the fact that only one lane in the gel was scanned.

5.4 DISCUSSION

Many hypotheses have been advanced to explain the mechanism of PHT-GH, including ascorbic acid deficiency (Kimball, 1939), adrenocortical derangement (Staple, 1953a and 1953b), hypersensitivity (Billen et al., 1964; Van der Kwast, 1956; Angelopoulos, 1975), local action of breakdown products of PHT (Brandon, 1948), and a direct action on fibroblasts (see Introduction). In this paper, we focused the study of drug-associated gingival overgrowth on the effects of PHT or NF on gingival fibroblasts, especially in regards to collagen catabolism.

The therapeutic concentrations in serum for PHT and NF are 10 to 30 µg/mL and 0.025 to 0.1 µg/mL, respectively (Drug: Facts and Comparison, 1987). The concentration of NF used in this study was twice as high as the therapeutic concentration. However, no toxicity to fibroblasts was found during the experiment. Our results showed that PHT and NF at concentrations 10 µg/mL and 0.2 µg/mL, respectively, had no effect on the proliferation of fibroblasts, like CsA at therapeutic concentrations (Chapter II). This does not agree with reports by Shafer (1960) and Zebrowski et al. (1988). The reason for these different results is not clear.

The effects of both NF and PHT on suppression of the degradation of fibrillar collagen by normal gingival fibroblasts were cell strain dependent and it was usually weaker than CsA, but no effect was found on the two CsA-GH responder cell strains tested. This indicates that CsA-GH cell strains are not sensitive to NF or PHT.

The effects of NF or PHT on ConA-stimulated collagenase production were not consistent in the 3 cell strains studied. NF either decreased or did not affect the ConA-induced collagenase production, whilst PHT either had no effect or enhanced the ConA effect. In one cell strain the fibroblasts produced measurable collagenase without stimulation with ConA. NF or PHT inhibited the "auto-release" of collagenase activity, but this effect was overcome by ConA-stimulation. This is again in conflict with the reports from Zebrowski et al (1988).

Since we do not have any NH- or PHT-GH responder cell strains, it is not appropriate to compare our results with those presented by Hassell (1982), who showed that collagenase secreted by PHT-GH responder fibroblasts was inactive. However, so far we have seen only little biological difference between fibroblasts from CsA-GH responder, non-responder, or normal cell strains in in vitro studies, i.e., NF and PHT did not suppress the degradation of fibrillar collagen by fibroblasts from CsA-GH cell strains. The negative result of Hassel (1982) on collagenase activity assay was probably due to the failure to add a collagenase-stimulating agent to the culture system (see Chapter IV).

In previous studies it was shown that CsA inhibited fibrillar collagen degradation in all the cell strains tested. CsA also depressed ConA-, or human recombinant interleukin-1(α)-induced collagenase production in all cell strain measured Chapter IV). Hence, in the presence of a stimulating agent, CsA has a more profound effect on suppressing the collagen catabolism than does NF or PHT. However, in the absence of a collagenase-stimulating agent, CsA does not inhibit procollagenase production if there is any, while NF or PHT do directly suppress this process.

In spite of the great similarity of these three drug-associated gingival hyperplasias in histopathology, we failed to show that they have a similar action mode in fibroblast metabolism of collagen by these in vitro studies. Comparison of the results from this paper with our previous ones on CsA, suggests that CsA, NF and PHT at therapeutic concentrations are not mitogenic to gingival fibroblasts. Their effects on inhibition of fibroblast catabolism of collagen differ in degree: CsA has the greatest effect, while the other two are cell-strain dependent in the presence of a stimulating agent. CsA-GH cell strains are not sensitive to NF or PHT.

5.5 BIBIOGRAPHY

ANGELOPOULOS, A.P. Diphenylhydantoin gingival hyperplasia. A clinicopathological review. I. Incidence, clinical features and histopathology. J. Can. Dent. Assoc. 41:103-106, 1975.

BALLARD, J.B., BUTLER, W.T. Proteins of the periodontium. Biochemical studies on collagen and non-collagenous proteins of human gingiva. J. Oral Pathol. 3: 176-184, 1974.

BARAK, S. ENGELGERG, I.S., HISS, J. Gingival hyperplasia caused by nifedipine. Histopathologic findings. J Periodontol. 58:639-642, 1987.

BENVENISTE, K., BITAR, M. Effects of phenytoin on cultured human gingival fibroblasts. In: Phenytoin-induced teratology and gingival pathology. T.M. Hassell, M.C. Johnston, K.H. Dudley, editors. Raven Press, N.Y. 1980. pp. 199-211.

BILLEN, J.R., GRIFFIN, J.W., WALDRON, C.A. Investigations for pyronine bodies and fluorescent antibodies in 5;5-diphenylhydantoin gingival hyperplasia. Oral Surg. Oral Med. Oral Pathol. 13:773-782, 1964.

BRANDON, S.A. treatment of hypertrophy of gingival tissue caused by Dilantin sodium therapy. J. Am. Dent. Assoc. 37:732-734, 1948.

BUTLER, R.T., KALKWARF, K.L., KALDAHL, W.B. Drug-induced gingival hyperplasia: phenytoin, cyclosporine, and nifedipine. J. Am. Dent. Assoc. 114:56-60, 1987.

DRUG: Facts and Comparison. E.K. Kastrup (founding editor). J.B. Lippincott Company, St. Louis, Missouri, 1987 edition. pp. 520 and 1121.

HASSELL, T.M. Evidence for production of an inactive collagenase by fibroblasts from phenytoin-enlarged human gingivae. J. Oral Pathol. 11:310-317, 1982.

HASSELL, T.M., PAGE, R.C., NARAYANAN, A.S., COOPER, C.G. Diphenylhydantoin (Dilantin) gingival hyperplasia: Drug-induced abnormality of connective tissue. Proc. Natl Acad. Sci., USA. 73:2909-2912, 1976.

KANTOR, M.L., HASSELL, T.M. Increased accumulation of sulphated glycosaminoglycans in cultures of human fibroblasts from phenytoin-induced gingival overgrowth. J. Dent. Res. 62:383-387, 1983.

KIMBALL, O.P. The treatment of epilepsy with sodium diphenylhydantoinate. J. Am. Med. Assoc. 112:1244-1245, 1939.

LEDERMAN, D., LUMERMAN, H., REUBEN, S, FREEDMAN, P.D. Gingival hyperplasia associated with nifedipine therapy. Oral surg. Oral Med. Oral Pathol. 57:620-622, 1984.

MANIATIS, T., FRITSCH, E.F., SAMBROOK, J. (Editors): Molecular cloning: A laboratory mannual. Cold Spring Harbor Laboratory, 1982. page 468.

MORGAN, A.R., LEE, J.S., PULLEYBLANK, D.E., MURRAY, N.L., EVENS, D.H.Review: Ethidium fluorescence assay. Part I. Physiochemical studies. Nucleic Acids Research. 7:547-569, 1979.

NARAYANAN, A.S., MEYERS, D.F., PAGE, R.C. Regulation of collagen production in fibroblasts cultured from normal and phenytoin-induced hyperplastic human gingiva. J. Periodont. Res. 23:118-121, 1988

PANUSKA, H.J.; GORLIN, R.J.; BEARMAN, J.E.; MITCHELL, D.F. The effect of anticonvulsant drug upon the gingiva: A series of analyses of 1048 patients. J.

Periodontol. 31:336-344, 1960

SCHNEIDER, W.C. Determination of nucleic acids in tissues by pentose analysis. In: Methods in Enzymology, Sidney P. Colowick and Nathan O. Kaplan, editors. Academic Press, N.Y., Vol.III, 1957. pp. 680-684.

SHAFER, W.G. Effect of dilantin sodium on growth of human fibroblasts-like cell cultures. Proc. Soc. Exp. Biol. Med. 104:198-201, 1960.

STAPLE, P.H. Diphenylhydantoin, adrenal function and epilepsy. J. Endocrinol. 9:18-20, 1953a.

STAPLE, P.H. The adrenal glands and gingival hyperplasia due to phenytoin sodium. Lancet. 265:600-601, 1953b.

VAN DER KWAST, W.A.M. Speculations regarding the nature of gingival hyperplasia due to diphenylhydantoin -sodium. Acta Med. Scand., 153:399-405, 1956.

ZEBROWSKI, E.J. RAMAMURTHY, N.S., SINGER, D.L., BRUNKA, J.R. Collagenase activity of cyclosporin, nifedipine and phenytoin treated fibroblasts. J. Dent. Res. 67 (Special issue):331, 1988.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

6.1 SUMMARY OF THE PREVIOUS STUDIES ON DRUG-ASSOCIATED GINGIVAL HYPERPLASIA

The findings of our studies on the effects of cyclosporine A (CsA), nifedipine (NF) or phenytoin (PHT) on gingival fibroblasts are as follows (see Chapter II, III, IV and V for detail):

(1) CsA, NF or PHT at therapeutic concentrations are not mitogens for gingival fibroblasts.

(2) CsA can slightly enhance gingival fibroblasts to phagocytose latex beads after 24 hours' incubation.

(3) CsA *per se* does not cause calcium influx nor does it interfere with the calcium ionophore A23187-induced calcium influx.

(4) CsA can rapidly pass through the fibroblast membrane and is highly concentrated in the cytosolic fraction, probably by the help of the intracellular binding molecule(s).

(5) The high affinity CsA-binding site in the cytosol has a dissociation constant (K_d) of 2.6×10^{-7} M, which is saturable when extracellular [CsA] is ≥ 1.0 µg/mL. The K_d for low affinity binding, which is non-saturable at [CsA] ≥ 1 µg/mL, is too high to be determined.

(6) CsA inhibits the degradation of fibrillar collagen by fibroblasts; it also suppresses the production of collagenase in response to stimulation by agents such as concanavalin A (ConA) and human recombinant interleukin-1α (hrIL-1α). However, CsA does not inhibit the effect of hrIL-1ß. CsA may possibly bind to hrIL-1α but not hrIL-1ß extracellularly and hence selectively depress the function of hrIL-1α.

(7) In comparing the effect of CsA on the catabolism of collagen by fibroblasts with NF or PHT, CsA causes more markely inhibits of degradation. Furthermore, the cells from CsA-GH tissue are not sensitive to NF or PHT suppression of the breakdown of fibrillar collagen. However, CsA only depresses some stimulating agent-induced collagenase production, while NF or PHT can directly inhibit the production of collagenase by the cells.

Our results do not support the idea that these three drugs may have a common molecular action mechanism in the catabolism of collagen by fibroblasts. Although we did not prove that CsA interferes with $[Ca^{2+}]i$ (intracellular free calcium ion concentration) in the above study, the possibility that $[Ca^{2+}]i$ changes are related to the regulation of collagenase can not be excluded (Unemori and Werb, 1988).

In conclusion, the direct effect of one of these drugs on gingival fibroblast is probably primarily responsible for the side-effect of the drug-associated gingival hyperplasia. Our results suggest that CsA most likely acts intracellularly rather than on the cell membrane. At a certain level, increase of collagen synthesis *in vivo* may act as a feedback signal to stimulate the production of collagenase. Therefore, the suppression of the degradation of fibrillar collagen by CsA, NF or PHT seen *in vitro* may be of physiological significance. The inhibition of some of the stimulatory agent-induced collagenase production by CsA, NF or PHT could be one of the major contributions to the drug-associated gingival hyperplasia. It further suggests that local inflammatory mediators *in vivo*, which can both increase synthesis of collagen and collagenase (Duncan and Berman, 1989) and thus accelerate the gingival tissue metabolism, are very important to the side-effect.

6.2 FUTURE TRENDS

6.2.1 The direct effects of cyclosporine A, nifedipine or phenytoin on gingival fibroblasts.

In the direct effects of CsA, NF or PHT on gingival fibroblasts, more work can be done in the field of collagen metabolism, especially in regards to the precise action mode of each of these drugs on the synthesis and degradation of collagen. The cell membrane transduction system as well as the intracellular signals related to the regulation of collagen metabolism need to be studied. From the pharmacology and previous experimental findings for these drugs (for a review, see Chapter I), the questions that can be directly asked are: If CsA acts intracellularly, is it via cyclophilin, calmodulin or other intracellular receptor? What is the physiological function of cyclophilin or calmodulin in relation to collagen metabolism? It was found that cyclophilin was identical to the enzyme PPI (peptidyl prolyl cis/trans isomerase) (Schmid et al., 1989), which is universal and non-specific in the cytoplasm. Thus, whether the binding of CsA to cyclophilin specifically affects the synthesis of collagen and/or collagenase is uncertain. To answer these questions, the high affinity intracellular binding molecule for CsA should be purified and its biochemical and physiological properties studied. For NF and PHT, the questions are: Does NF affect the calcium channel of fibroblasts? Does PHT interfere with the Na^+-K^+, Ca^{2+} and other ion flux in fibroblasts? Do all or any of these observations relate to the regulation of collagen metabolism?

Based on the previous studies, two more interesting questions can be raised: (a) Except for collagen fibrils, especially in the situation of gingival inflammation, what are other collagenase-stimulating agents in vivo? (b) Do fibroblasts from CsA-GH, NF-GH and PHT-GH behave differently in response to the collagenase stimulation with physiologically relevant agents other than fibrillar collagen and IL-1?

6.2.2. The contribution of other factors to the drug-associated gingival hyperplasia.

In addition to the direct effect of the drug on fibroblasts, other indirect factors may contribute to these drug-associated gingival hyperplasias. Among them, epithelial cells, inflammatory mediators and the immune system all need to be researched.

For the epithelial cells, the questions are: Does CsA, NF or PHT stimulate the proliferation of epithelial cells? Do these epithelial cells produce less collagenase than the non-drug-treated cells in the situation of gingivitis?

Connective tissue in gingivitis and periodontitis is usually seen in the process of degradation rather than overgrowth (Shafer et al., 1983) in the non-CsA-, non-NF-or non-PHT-treated patients. Unlike drug-induced GH, gingiva swelling during gingivitis is mainly due to inflammatory filtration and edema. Even in the so called inflammatory-induced "gingival hyperplasia", the histopathological features only show a non-specific chronic inflammation (Shafer et al., 1983) rather than the true fibrous hyperplasia. Then why does connective tissue in drug-associated GH show an increase of collagen content? It is obvious that the direct effect of CsA, NF, or PHT on the suppression of the degradation of fibrillar collagen, production of collagenase by fibroblasts (see above) and the inhibition of the production of IL-1 (at low [CsA] 50 ng/mL, but not at higher [CsA] 100 to 150 ng/mL) by macrophages (Tipton et al., 1989) could greatly contribute to the collagen accumulation in the side-effect of these drugs. Whether other factors such as the effects of immune cells (especially plasma cells) can play a role in the drug-associated GH is unknown. Does immunoglobin (such as IgG) increase in the gingival tissue? Can the IgG itself or antigen-immunoglobin complex stimulate the production of collagenase in vivo? If so, do CsA, NF or PHT depress their effect?

There is a report that the decrease of collagenase activity is a consequence of reduction of IL-1 production in CsA-treated monocyte in the presence of lipopolysaccharide (LPS) stimulation (Tipton et al., 1989). We have observed that CsA can selectively inhibit the effect of IL-1α on the stimulation production of collagenase. Although CsA seems to be working extracellularly in this effect (Chapter IV), its physiological importance cannot be evaluated until information on the concentration of extracellular CsA in gingival tissue is available. It is known that CsA at 1 µg/mL does not affect mRNA levels of IL-1 in monocytes stimulated by LPS (10 ng/mL) (Granelli-Piperno and Keane, 1988). However, the ratio of IL-1α and IL-1ß produced in vivo by LPS-stimulated macrophages during CsA treatment needs to be investigated.

In summary, future work in the above fields can help us to have a better understanding of these drug-associated gingival hyperplasias and to know more about the molecular mechanisms which balance collagen metabolism in the physiological condition and its disturbance in pathological situations. It may also benefit the prevention and clinical management of these side-effects in the future.

6.3 BIBLIOGRAPHY

DUNCAN, M.R., BERMAN, B. Differential regulation of collagen, glycosaminoglycan, fibronectin, and collagenase activity production in cultured human adult dermal fibroblasts by interleukin 1 alpha and beta and tumor necrosis factor -alpha and beta. J. Invest. Dermatol. 92:699-706, 1989.

GRANELLI-PIPERNO, A., KEANE, M. Effects of cyclosporine A on T lymphocytes and accessory cells from human blood. Transplant. Proceedings, 20 Suppl. 2:136-142, 1988.

SCHMID, F.X., KIEFHABER, T., SCHÖNBRUNNER, R. The mechanism of slow steps in protein folding. In: The 19th Federation of European Biochemical Societies Meeting. Rome, July 2-9, 1989. Abstract No. TU 66 L.

TIPTON, D., PABST, M., DABBOUS, M. Effect of immune response on fibroblast collagenase activity. J. Dent. Res. 68 Special Issue:1433, 1989.

SHAFER, W.G., HINE, M.K., LEVY, B.M., TOMICH, C.E. A Textbook of Oral Pathology. 4th edition. W.B. Saunders Company, Philadelphia, 1983. pp. 772-794.

UNEMORI, E.N., WERB, Z. Collagenase expression and endogenous activation in rabbit synovial fibroblasts stimulated by the calcium ionophore A23187. J. Biol. Chem. 263:16252-16259, 1988.